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THE PITUITARY-GONAD RESPONSE OF GENETICALLY OBESE MICE IN PARABIOSIS WITH THIN AND OBESE SIBLINGS[1]

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ABSTRACT

Gonadectomized mice that were either obese or nonobese, male or female were parabiosed to either obese or nonobese immature female siblings. The experiment was designed (a) to compare the production of gonadotrophin by the pituitaries of the obese and nonobese gonadectomized animals and (b) to compare the relative responses of the reproductive systems of the obese and nonobese assay females to the gonadotrophin produced by the pituitary of the castrated partner. Results showed that, although the pituitaries of the gonadectomized obese mice of both sexes as well as those of the nonobese mice produced excess gonadotrophin, the obese animals elicited less ovarian hypertrophy in the assay female than did nonobese mice. Also, female animals elicited less response than males. The reproductive systems of the female obese and nonobese mice responded similarly to the excess gonadotrophin. It is concluded that, while the pituitaries of obese mice are competent to produce more gonadotrophin than they normally do, under the conditions of this experiment the pituitaries in obese mice were less competent than those in nonobese mice.

ALL genetically obese female mice homozygous for the autosomal recessive gene *ob* are sterile. The reproductive system of the obese female never matures; the uterus remains in a prepuberal state and ovulation never takes place. Investigators have shown, however, that both the ovaries and the ova of obese females can produce offspring when transplanted into the normal endocrine environment of a thin female mouse (1, 2). It has also been shown that the obese ovary in its own environment is capable of responding to exogenous gonadotrophins (2, 3). Not all obese male mice are sterile. Some obese males are capable of producing offspring when they are maintained at a body weight similar to that of nonobese males by restricted food intake (4). Other breeding experiments with un-

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treated obese males have shown that approximately 20% of these males are able to reproduce. All the above observations on the reproductive capacities of obese males and females suggest that the pituitary gland of the obese animal does not produce adequate gonadotrophin.

The use of parabiosis to study pituitary-gonad interrelations has been reviewed by Finerty (5), but several pertinent facts will be mentioned here. It is an established fact that when a castrated male or ovariectomized female rat or mouse is joined to an intact female the ovaries of the intact female hypertrophy. Ample evidence has been accumulated to show that this hypertrophy is caused by an excess of circulating gonadotrophin (probably FSH) from the pituitary of the gonadectomized partner. The increased weight of the ovaries then becomes an indicator of the circulating gonadotrophin produced by the pituitary of the castrate (6, 7). The technique of parabiosis was used in this experiment, 1) to determine if the pituitary gland of the obese mouse is capable of producing excess gonadotrophin as a response to castration and 2) to compare the relative responses of the reproductive systems of the obese and nonobese female mice to the gonadotrophin produced by the pituitary of the gonadectomized partner.

MATERIALS AND METHODS

All mice used in this experiment were littermate pairs obtained from the noninbred stock of obese mice of the Roscoe B. Jackson Memorial Laboratory. The operative procedure used was that described by Bunster and Meyer (8). One member of each pair was gonadectomized prior to joining and all pairs were joined at weaning or as soon after as the obesity was evident (23 to 33 days of age). Autopsies were performed on all parabiotic pairs that were in good health at 4 weeks after joining, when the animals were 8 to 9 weeks old. A 4 week period of parabiosis was utilized rather than the commonly used 11 days in order to detect and eliminate as much immunological incompatibility between co-twins as possible. Because the obese stock was not inbred some genetic incompatibility was expected to occur. Finerty and Panos (9) have shown that incompatibility is responsible for the death of parabiotic rats between the 13th and 18th post operative day. By using only healthy pairs of animals that have been in parabiosis for 28 days most of the incompatible pairs should be eliminated. In addition, a longer period of parabiosis might provide information concerning a possible hormonal mechanism for the action of the obese gene.

Each animal was weighed prior to joining and again at autopsy. Both ovaries of each intact female were observed, removed and weighed together on a Roller-Smith torsion balance and prepared for section. Uteri were blotted, weighed and saved in the same manner as the ovaries.

Eight experimental groups, consisting of 1 to 6 parabiotic pairs per group, were used. Four groups were made up of thin and obese castrated males and of thin and obese ovariectomized females each joined to an immature intact obese female mouse. The other 4 groups were made up of the same 4 types of gonadectomized animals each joined to an immature intact nonobese female mouse. The experiment was designed so that intact obese or nonobese females served as assay parabionts, and gonadectomized partners that were either obese or nonobese and male or female served as stimulating parabionts. The intact obese and nonobese females will hereafter be referred to as the assay parabionts. The gonadectomized partners will be referred to as the stimulating parabionts.

Two control groups, used in conjunction with another experiment, are included in order to compare ovarian and uterine weights of parabiosed females with those of single females.[2] These consisted of 14 single intact thin females and 14 single intact obese females autopsied at 8 to 9 weeks of age. The ovarian and uterine weights were obtained in the same manner as were those of the parabionts.

RESULTS AND DISCUSSION

Thirty-two pairs of parabiosed animals showed no signs of physiological incompatibility prior to autopsy. However, 8 (25%) of these, 6 obese assay females and 2 nonobese assay females, showed no ovarian stimulation and have been omitted from the analysis as functionally nonparabiotic. Investigators have previously reported (6, 10, 11) that 11 to 15% of parabiotic rats have failed to show gonadal stimulation. The lack of ovarian stimulation was judged by both the histological examination of the ovaries and the ovarian weights. The ovaries of these 8 assay females showed no large cystic or hemorrhagic follicles, which are typical of parabiotic ovaries. The smallest pair of ovaries, accompanied by ovarian hypertrophy, included in the statistical ananlysis weighed 8.3 mg. and the heaviest pair of ovaries, showing no evidence of hypertrophy, deleted from the analysis weighed 7.0 mg. The inclusion of these 8 pairs in the statistical analysis would, if anything, have strengthened the reported conclusions concerning the difference in stimulating capacity between both obese and nonobese and males and females.

Hausberger (12) has recently reported that weight gain is suppressed in obese mice maintained in parabiosis with thin siblings for 4 months. The mean gain in body weight for obese animals attached to obese siblings during the 4 week period of parabiosis was 11.66 gm.; for obese animals attached to nonobese siblings, 11.54 gm. The mean gain for nonobese animals attached to nonobese was 4.50 gm. and for nonobese attached to obese, 3.08 gm. In all instances where an obese animal was parabiosed with a thin littermate, the obese animal retained its obese appearance and in no instance did a thin animal become obese. There is thus no evidence that any circulating factor influencing obesity was effective during the 4 weeks these animals remained joined.

The ovarian histology showed that in all obese and nonobese assay females the stimulated ovaries were made up of numerous large cystic and hemorrhagic follicles. A few assay females of each genotype had 1 or 2 corpora lutea atretica and all showed some degree of follicular luteinization. No distinctive differences between the ovaries of the obese and nonobese assay females were noted. At the time of autopsy 6 obese and 6 nonobese females had reached a state of constant estrus, as indicated by vaginal cornification. Constant estrus in assay females had been produced by all 4 types of stimulating parabionts. In pairs made up of obese stimulating fe-

[2] Courtesy of Dr. Meredith N. Runner.

TABLE 1. COMPARISON OF MEAN OVARIAN AND UTERINE WEIGHTS OF OBESE AND NONOBESE MICE WITH AND WITHOUT PARABIOSIS

Genotype of mice	Mean ovarian weight* ± S E				Mean uterine weight* ± S E			
	Parabiosed	(No.)	Nonparabiosed	(No.)	Parabiosed	(No.)	Nonparabiosed	(No.)
Obese	21.53 ± 2.45	(11)	4.50 ± 0.38	(14)	118.92 ± 14.40	(11)	8.74 ± 0.73	(14)
Nonobese	23.81 ± 3.50	(13)	7.20 ± 0.48	(14)	116.42 ± 9.61	(13)	77.56 ± 6.94	(14)
Probability	> .05		< .01		> .05		< .01	

* Mg.

males joined to obese assay females, sufficient gonadotrophin production was combined with sufficient ovarian responsiveness to produce a state of constant estrus in the obese assay female. It can be stated on the basis of ovarian histology and induction of vaginal cornification that obese parabionts served effectively both as the assay partners and as the stimulating partners.

The effect of the gene for obesity on the ovarian and uterine weights of single intact young females if shown in Table 1. The weights of these organs are significantly less in obese than in nonobese mice. That parabiosis causes a tremendous increase in ovarian and uterine weight in both obese and nonobese females is also shown in Table 1 where the data from animals in parabiosis is summarized. These data demonstrate that the ovary of the obese female is capable of elaborating gonadal hormones in response to the excess gonadotrophin produced by the pituitary of the attached castrate. This suggests that if ample gonadotrophin were available from the obese female's own pituitary, maturation of its reproductive system could occur.

The results of the parabiosis experiment are shown in Table 2 where the means and standard errors of the ovarian weights for each experimental group are presented. Since uterine weights were highly variable only ovarian weights have been considered. The numbers of pairs of parabiotic mice

TABLE 2. MEAN WEIGHTS OF PAIRS OF OVARIES FROM ASSAY PARABIONTS (MG.)

Genotype of parabiont		Sex of stimulating parabiont					
		Male		Female		Total	
Assay	Stimulating	No.	Mean S.E.	No.	Mean S.E.	No.	Mean S.E.
Obese	Obese	2	24.50 ± 0.16	4	15.07 ± 3.04	6	18.20 ± 2.77
	Nonobese	1	22.20 —	4	26.35 ± 4.77	5	25.52 ± 3.76
	Total	3	23.73 ± 0.82	8	20.71 ± 3.37	11	21.53 ± 2.45
Nonobese	Obese	3	23.80 ± 5.83	6	15.95 ± 2.27	9	18.57 ± 2.58
	Nonobese	2	47.10 ± 3.91	2	24.15 ± 5.87	4	35.62 ± 7.21
	Total	5	33.12 ± 6.64	8	18.00 ± 2.40	13	23.81 ± 3.50
Total	Obese	5	24.08 ± 3.24	10	15.60 ± 1.68	15	18.42 ± 1.84
	Nonobese	3	38.80 ± 8.34	6	25.61 ± 3.41	9	30.01 ± 3.97
	Total	8	29.60 ± 4.34	16	19.36 ± 1.93	24	22.77 ± 2.22

in some of the cells are smaller than would ideally have been desirable, but since, as will be seen (Table 3), there are no significant interactions among the 3 factors (sex and genotype of the stimulating parabiont, and genotype of the assay parabiont) the small samples are adequate to establish the observed significant differences. The significance of the differences between the means of the various groups was assessed by the use of an analysis of variance. The mean squares and F values for each source of variance are given in Table 3. The mean ovarian weights (see Table 2) produced by the nonobese stimulating parabionts (30.01 ± 3.97) are significantly greater than those produced by the obese stimulating mice (18.42 ± 1.84). The mean ovarian weights produced by the male stimulating parabionts (29.60 ± 4.24) are significantly greater than those produced by the females (19.36 ± 1.93). The mean ovarian weights of the obese assay females (21.53 ± 2.45) do not differ significantly from those of the nonobese assay

TABLE 3. ANALYSIS OF VARIANCE OF OVARIAN WEIGHT IN PARABIOTIC MICE

Source of variance		d.f.	Mean square	F	P
Main Effects					
Genotype of Assay Female	(A)	1	30.94	—	
Genotype of Stimulating Parabiont	(G)	1	754.87	15.23	$<.01$
Sex of Stimulating Parabiont	(S)	1	559.65	11.29	$<.01$
Interactions					
A×G		1	196.40	3.96	$>.05$
A×S		1	163.69	3.30	$>.05$
G×S		1	12.14	—	
Error		17	49.56	—	

females (23.81 ± 3.50). These results show that although obese mice do produce additional gonadotrophin in response to castration they elicit less ovarian hypertrophy in the assay female than nonobese mice do. It is evident, by the competence with which the obese ovary responds to exogenous gonadotrophin (2, 3) or a normal endocrine environment (1), that the pituitary gland of the obese mouse normally does not produce adequate gonadotrophin. That the obese pituitary also produces less gonadotrophin than the nonobese as a response to castration has been shown by parabiosis.

Previous investigators (6, 10, 11) using parabiosis for 11 days have found no difference in the ovarian weights betwen castrated male-female pairs and ovariectomized female-female pairs. However, these investigators and others (13) have shown that greater amounts of gonadal hormones are needed to suppress the pituitary hypersecretion of gonadectomized male rats than that of gonadectomized female rats. Thus, there is some difference between male and female pituitary gonadotrophins in rats, even though it is not measurable by ovarian weight after 11 days of parabiosis. The experiments presented here show that female mice elicit less response than males and that the difference is demonstrable by ovarian weight after 4 weeks of parabiosis. The difference in the fertility of obese males and fe-

males suggested a possible sex difference in pituitary secretion in addition to a common hyposecretion on low potency of gonadotrophin associated with genetic obesity. The evidence in this paper supports this conclusion.

The competence of the ovary of the obese female mouse to respond to gonadotrophin from its partner compares favorably with that of the nonobese female. This agrees with the results found by other investigators (2, 3).

It is, therefore, concluded that while the pituitaries of obese mice are competent to produce more gonadotrophin than they normally do, under the conditions of this experiment the pituitaries in obese mice were less competent than those in nonobese animals. This suggests that the sterility observed in all obese female mice is the result of a hypofunction of the pituitary associated with genetic obesity and not the result of an impaired function of the reproductive organs.

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REFERENCES

1. Hummel, K. P.: *Anat. Rec.* **128:** 569. 1957.
2. Runner, M. N. and A. Gates: *J. Hered.* **45:** 51. 1954.
3. Runner, M. N.: *Genetics* **39:** 990. 1954.
4. Lane, P. W. and M. M. Dickie: *J. Hered.* **45:** 56. 1954.
5. Finerty, J. C.: *Physiol. Rev.* **32:** 277. 1952.
6. Meyer, R. K. and R. Hertz: *Am. J. Physiol.* **120:** 232. 1937.
7. Meyer, R. K., C. Biddulph and J. C. Finerty: *Endocrinology* **39:** 23. 1946.
8. Bunster, E. and R. K. Meyer: *Anat. Rec.* **57:** 339. 1933.
9. Finerty, J. C. and T. C. Panos: *Proc. Soc. Exper. Biol. & Med.* **76:** 833. 1951.
10. Biddulph, C., R. K. Meyer and L. G. Gumbeck: *Endocrinology* **26:** 280. 1940.
11. Hertz, R. and R. K. Meyer: *Endocrinology* **21:** 756. 1937.
12. Hausberger, F. X.: *Anat. Rec.* **130:** 313. 1958.
13. Bunster, E. and R. K. Meyer: *Endocrinology* **23:** 496. 1938.

FURTHER STUDIES OF THE HORMONAL INDUCTION OF MELANOGENESIS IN THE KILLIFISH, *FUNDULUS HETEROCLITUS*

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ABSTRACT

New experiments confirm the respective roles of prolactin and intermedin in the *in vivo* melanin pigmentation of *Fundulus heteroclitus:* prolactin promotes melanin synthesis, intermedin stimulates proliferation of melanocytes. Prolactin potentiates the response to intermedin, but intermedin has no reciprocal influence on the response to prolactin. Treatment with alkali enhances the activity of intermedin about tenfold. The response to alkali-treated intermedin increases in proportion to the dose. The slight melanocyte stimulating action of ACTH, which is potentiated by prolactin, is concordant with its inherent MSH-like activity.

In vitro studies of fin samples incubated with dopa indicate a significant correlation between O_2 consumption and degree of darkening. After hypophysectomy, which leads to progressive *in vivo* depigmentation, dopa tyrosinase activity declined. Enzyme activity was restored, to a greater or lesser extent, by chronic administration of any of the 3 hormones under investigation: prolactin, intermedin, and ACTH. Only prolactin restored *in vivo* melanin pigmentation, possibly by increasing the supply of endogenous precursor. Since new pigment cells are being formed, the stimulating action of intermedin on the enzyme system may be different from that of prolactin in which only an intracellular increase in already existing cells can be postulated. The influence of prolactin in potentiating the response to intermedin may be correlated with its hypothetical action on chromagen precursor. ACTH appears to stimulate both dopa tyrosinase and also a non-tyrosinase oxidative enzyme system.

PREVIOUS investigations (1) have shown that two pituitary hormones participate in the development of melanin pigmentation in Fundulus. Intermedin stimulates melanocyte proliferation and its action is potentiated by prolactin; prolactin promotes an increase in melanin synthesis without new pigment cell formation.

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The experiments reported in the present contribution are concerned with the relationship of these responses to the dopa tyrosinase enzyme system involved in melanin synthesis. Lerner (2) has reviewed some of the problems involved and a recent article by Foster (3) contains material relevant to the discussion. Investigations of tyrosinase and "dopa oxidase" activity in fishes have been confined to the study of genetic color mutants (4–7) and melanoma tissue (7–10); however, the basic problems are similar. Chronic treatment with effective hormones might influence one or more facets of the melanogenic sequence: the nature and amount of enzymes present, the availability of chromagen precursors, the presence of enzyme inhibitors, and conditions prerequisite for a possible non-oxidative terminal phase of darkening. Furthermore, an increase in O_2 consumption upon incubation with a melanin precursor cannot be taken *per se* as evidence for stimulation of the tyrosinase system. It is necessary to demonstrate a concomitant increase in pigment synthesis, or employ a specific tyrosinase inhibitor.

MATERIALS AND METHODS

The experiments were made on normal and hypophysectomized male *Fundulus heteroclitus* captued in the vicinity of New Haven, Connecticut. Care of the fish and experimental procedures were similar to those used in previous studies (1). Weights, standard lengths, and nuptial coloration were recorded at the time of hypophysectomy, at the beginning of the experiment and at autopsy. A period of at least 6 weeks was allowed to elapse after removal of the pituitary and any fish which increased more than 1 mm. in length or showed nuptial coloration was rejected. Intraperitoneal injections were made thrice weekly for periods of 4–7 weeks; the volume injected was kept constant at 0.01 ml. per gm. weight of fish. Autopsies were made on the day following the last injection. The tails and dorsal fins were taken for assays of melanin and dopa tyrosinase. Testes were weighed and expressed as percentage body weight (gonosomatic index). In Exp. 4 the right head kidney containing adrenal cortical tissue was fixed in Zenker's fluid, sectioned, and stained with hematoxylin and eosin or by the Azan method.

During the course of each experiment the bellies were examined at frequent intervals under a dissecting microscope to detect the appearance of new pigment cells. In Exp. 6 and 7 especially careful observations were made on each injection day, with sketches to indicate the appearance or disappearance of pigment cells.

Melanin assays were made as described previously, by a modification of the method of Foster, Cook and Stamas (11). In Exp. 1, 2 and 3 the data were derived from the unincubated control for the dopa tyrosinase study and represent pooled fin samples from each experimental group. In subsequent experiments a part of the tail fin of each fish was fixed separately in alcohol-formalin (9 parts 95% alcohol: 1 part formaldehyde), washed in 80% alcohol, decalcified with 3% HNO_3 in 70% alcohol (3 days), dehydrated through 80%, 95%, and 100% alcohol, washed with two changes of ether and dried. Decalcification was omitted in Exp. 1, 2 and 3, and in Exp. 6 the fin samples were kept frozen for 8 months before fixation. Weighed amounts of the dried fins were minced and extracted with thioglycolate solvent in a water bath at approximately 98° C (1 mg. per 1 ml. solvent). The mixture was allowed to cool for one hour and the amount of pigment extracted was estimated in Klett units.

Two criteria were employed for the estimation of dopa tyrosinase activity: the ability

of the tissue to darken when incubated with dopa and the increase in oxygen uptake. Pilot experiments showed that body skin gave negative results, in agreement with the findings of Wallbrunn (6) on *Betta splendens*. The difficulty may reside in the abundance of mucous cells in the epidermis. Enzyme tests were therefore restricted to fin samples. Furthermore, preliminary tests with tyrosine were negative. Frozen lyophilized fin material was incubated in a Warburg respirometer, using 1.5 ml. of reactants in 10 ml. flasks at 38° C. Two samples from each experimental group were used: one incubated in M/10 phosphate buffer (pH 6.8), the other in L-dopa (Delta Chemical Works) at a concentration of 0.5 mg. per ml. buffer. Readings were made over a period of 5 hours or more. After incubation, the residual fin material was fixed in alcohol-formalin, washed in 100% alcohol, cleared in toluol, and dried after soaking in ether. Melanin assays were performed as previously, on buffer incubated, dopa incubated, and unincubated samples.

The following hormones were employed:

Prolactin: 1. Armour Lot 759–39 CCC sheep prolactin (25–30 I.U./mg.) containing 0.02–0.03 I.U./mg. ACTH (courtesy of Dr. Sanford L. Steelman). An assay for frog melanophore dispersing activity by Dr. M. R. Wright showed 14×10^4 MSH units/gm. 2. Schering Hyex 4, sheep prolactin (ca. 35 I.U./mg.). As reported previously (1), this preparation contained 13.75×10^4 MSH units/gm. and sufficient ACTH to stimulate the adrenal cortical tissue of hypophysectomized Fundulus. 3. Three unnumbered lots of highly purified sheep prolactin (C. H. Li) with an assay of 35 I.U./mg. This material was both MSH and ACTH free.

Alpha adrenocorticotropin: Sheep alpha (α_s)−ACTH, 150 I.U./mg. (C. H. Li). The frog melanophore dispersing activity of this preparation, reported by Dr. M. R. Wright, was 4.4×10^7 MSH units/gm.

Intermedin: Three preparations of highly purified pig β-MSH were employed, through the courtesy of Dr. I. I. Geschwind. 1. Lot CCD-4T had an assay of ca. 4×10^9 MSH units/gm. (Dr. Geschwind gave $4–5\times10^9$ and Dr. Wright's later determination was in essential agreement, $3–4\times10^9$.) 2. The same material but treated with alkali and containing 33.3% salt. In preparing solutions for injection a correction was made for the salt content. 3. An alkali-treated salt-free preparation with an assay of 1×10^9 MSH units/gm. (I. I. Geschwind).

Prolactin solutions were prepared in advance and frozen in daily quota. The dose was the same in all experiments: 10 μg. per gm. weight of fish. In Exp. 5 the non-alkali treated intermedin and the combination of prolactin and intermedin were also prepared in advance, as was α_s-ACTH in Exp. 4. In Exp. 6 and 7 the MSH-containing solutions were freshly prepared immediately before use on each injection day. The doses are given in Table 1.

RESULTS

General: Weight and length changes showed that none of the hormone preparations had any significant growth promoting action on hypophysectomized recipients. ACTH caused an unusually severe weight loss and a mean length decrease of the order of 1% which was not statistically significant. The normal fish employed in Exp. 1 and 2 also failed to grow, but these experiments were made in late winter and early spring before the onset of the natural growing season. The gonosomatic index of hypophysectomized and hypophysectomized-injected fish was uniformly low. In Exp. 6 there was a slight but significant increase at the highest dose of untreated intermedin; no such stimulation was seen with a similar dose of the alkali-

TABLE 1. MELANOCYTE PROLIFERATION AND *in vivo* MELANIN SYNTHESIS IN RESPONSE TO PROLACTIN, MSH AND ACTH

Exp. No.	Condition[1]	Duration weeks	Hormone[2] or control	Dose per gm.	No. of fish[3]	New melanocytes[4]						Melanin			
						0	±	+	++	+++	++++	KU[5]	*s*	Diff. %[6]	Method[7]
1	Normal	5	None	—	8	8	—	—	—	—	—	42.3*	1.4	41.9	A
	H (3 mos.)	5	None	—	7[8]	7	—	—	—	—	—	29.8	2.2	—	A
	H (3 mos.)	5	Prol. (A)	10 μg.	8	4	3	1	—	—	—	39.9	3.9	34.1	A
2	Normal	7	None	—	9	9	—	—	—	—	—	45.0	—	11.1	B
	H (6 wks.)	7	None	—	5	5	—	—	—	—	—	40.5	—	—	B
	H (6 wks.)	7	Prol. (L)	10 μg.	6	6	—	—	—	—	—	39.5	—	−2.5	B
3	H (7 wks.)	4	None	—	7	7	—	—	—	—	—	49.3	—	—	B
	H (7 wks.)	4	Prol. (S)	10 μg.	8	6	1	—	1	—	—	54.6	—	12.5	B
4	H (7 wks.)	4	None	—	10	10	—	—	—	—	—	86.0	11.5	—	C
	H (7 wks.)	4	Prol. (L)	10 μg.	10	10	—	—	—	—	—	103.0*	15.3	19.8	C
	H (7 wks.)	4	α_s-ACTH	0.45 I.U.[9]	10	7	3	—	—	—	—	98.0	16.1	14.0	C
	H (7 wks.)	4	Combination		10	—	2	3	2	3	—	113.0**	18.3	31.4	C
5	H (8 wks.)	4	0.6% NaCl	0.01 ml.	10	10	—	—	—	—	—	91.7	7.1	—	C
	H (8 wks.)	4	Prol. (L)	10 μg.	10	10	—	—	—	—	—	100.4	11.0	9.5	C
	H (8 wks.)	4	MSH (1)	(6000 U.)[10]	9[8]	9	—	—	—	—	—	92.3	11.3	0.7	C
	H (8 wks.)	4	Combination		10	—	—	—	1	9	—	115.9**	13.4	26.4	C
6	H (10 wks.)	4	MSH (1) and MSH (2)-OH			See Table 2						See Table 2			D
7	H (7 wks.)	4	0.6% NaCl	0.01 ml.	11	11[11]	—	—	—	—	—	82.2	11.8	—	C
	H (7 wks.)	4	Prol. (L)	10 μg.	12	12[11]	—	—	—	—	—	95.5*	12.5	16.2	C
	H (7 wks.)	4	MSH (3)-OH	8000 U.	10	—	—	—	4	5	1	86.3	14.9	5.0	C
	H (7 wks.)	4	Combination		10	—	—	—	—	—	10	100.8**	17.6	22.9	C

[1] Normal = intact unoperated controls. H = hypophysectomized, time after hypophysectomy in parentheses ( ).
[2] Prol. = prolactin, (A) Armour, (L) C. H. Li, (S) Schering. MSH (1) = non alkali treated intermedin, MSH (2)-OH and MSH (3)-OH = alkali treated preparations. See text for details.
[3] Males unless otherwise indicated.
[4] 0 = no proliferation; ± = a few new melanocytes, usually in small clusters in front of each pelvic fin; + = new melanocytes sparsely dispersed over posterior half of belly; ++ = new melanocytes dispersed over whole of belly, following pattern of scale pockets; +++ = belly more densely pigmented, with obliteration of scale pattern; ++++ = belly nearly as black as dorsal surface.
[5] KU = Klett units; *s* = standard deviation.
[6] Per cent difference from hypophysectomized controls.
[7] A = assays on pooled fin samples frozen at autopsy, subsequently thawed and fixed but not decalcified; mean and standard deviation based on 4 samples per group. B = the same, but only one sample per group, therefore no statistics. C = assays on individual fin samples fixed at autopsy and subsequently decalcified. D = assays on individual samples frozen at autopsy, subsequently fixed and decalcified.
[8] One female included by mistake.
[9] MSH activity = 132 U./gm. of fish.
[10] Solution stored frozen, with apparent loss of potency (see text).
[11] Exact observations revealed minor fluctuating changes (see text).
* Significantly different from hypophysectomized controls.
** Significantly different from hypophysectomized controls, but not significantly different from prolactin treated controls.

treated preparation. ACTH administered alone or in conjunction with prolactin caused a marked stimulation of the adrenal cortical tissue with an increase in the number of cell layers and extensive proliferation into the adjoining leucopoietic tissue of the head kidney.

New melanocytes (Tables 1 and 2): Earlier experiments indicated that fluctuating minor changes might occur in the hypophysectomized controls

TABLE 2. INFLUENCE OF DOSE AND ALKALI TREATMENT ON THE MELANOCYTE PROLIFERATING ACTION OF INTERMEDIN

Hormone[1]	Dose per gm. wt.	New melanocytes				Melanin		
		Week 1	Week 2	Week 3	Week 4	KU	Mean	*s*
MSH (1)	4,000 I.U.	−	−	−	−	128.5		
		−	−	−	−	116.5		
		−	−	−	±	127.5		
		−	±	±	±	105.5		
		−	+	+	++	139.5	123.5	12.9
	12,000 I.U.	−	−	−	±	120.5		
		−	−	−	±	165.5		
		−	±	±	±	160.5		
		−	±	±	+	118.5		
		−	+	+	+	124.5	137.9	23.1
	40,000 I.U.	−	−	−	−	104.5		
		−	−	±	±	99.5		
		−	±	±	±	131.5		
		−	±	±	±	115.5		
		−	+	++	++	146.5	119.5	19.5
MSH (2)-OH	4,000 I.U.	−	−	−	−	134.5		
		−	±	±	±	140.5		
		−	±	+	+	142.5		
		−	+	++	++	114.5		
		−	+	++	+++	129.5	132.3	11.2
	12,000 I.U.	−	±	+	+	117.5		
		±	±	+	+	140.5		
		+	+	++	++	159.5		
		±	+	++	+++	111.5		
		++	++	++	+++	136.5	133.1	19.2
	40,000 I.U.	±	+	++	++	174.5		
		±	++	+++	+++	162.5		
		+	+	++	+++	117.5		
		+	++	+++	++++	136.5		
		+	++	+++	++++	163.5	150.9	14.9

[1] See Table 1 for explanation of symbols and further information.

and the careful records for Exp. 7 confirmed this suspicion. Minute new pigment cells occasionally appeared and often disappeared again; possibly some of these were pigment-laden macrophages. Melanophores that had been present at the start of the experiment sometimes disappeared or changed their position; sometimes a pigment cell disintegrated into numerous small fragments.

All prolactin preparations restored, or partially restored, the melanin pigment in ghosts of large melanophores that had faded as the result of

** Significantly different from hypophysectomized controls, but not significantly different from prolactin treated controls.

hypophysectomy. Such cells, barely visible at the start of the experiment, became conspicuous after the second or third injection. On the other hand, highly purified prolactin (C.H.Li) never resulted in the proliferation of new melanocytes, although minor fluctuating changes similar to those recorded for the controls were observed in Exp. 7 and presumably occurred in earlier experiments. The Schering and Armour preparations, both known to contain a trace of MSH-like activity, caused some new pigment cell formation: 4 out of 8 advanced to the ± or + stage with Armour prolactin; 2 out of 8 achieved the ± and ++ stage respectively with Schering prolactin.

The findings with intermedin depended on the dose, on whether the material had been treated with alkali, and on whether the injection solution was freshly prepared immediately before use. The intermedin solution which had been prepared and frozen in advance (Exp. 5) gave negative results, although the same material prepared in conjunction with prolactin showed the expected order of activity. Exp. 6, in which untreated and alkali-treated intermedin were compared at three dosage levels, showed a graded series of results (Table 2). The untreated preparation elicited some proliferation of new pigment cells, scarcely greater at the highest than at the lowest dose. Alkali-treated intermedin caused considerable melanocyte proliferation at the lowest dose, approximately equivalent to the response at the highest dose of untreated hormone. The highest dose of alkali-treated intermedin elicited a very striking response. The results suggest that alkali-treatment potentiates the action about ten-fold and that the magnitude and speed of onset of the response is a function of the dose.

Combination of intermedin and prolactin (Exp. 7) gave the expected results: a striking increase in the melanocyte-proliferating action of intermedin.

ACTH caused a slight proliferation of new pigment cells in 3 out of 10 recipients (Exp. 4). The combination with prolactin greatly enhanced the response and 5 out of 8 recipients advanced to the ++ stage or beyond.

Melanin (Tables 1 and 2): The absolute values of the melanin assay are only comparable between one experiment and another when the pretreatment of the fin samples was identical. Lack of decalcification seems to have been responsible for the lower readings in Exp. 1, 2 and 3 as compared with Exp. 4, 5 and 7.

After removal of the pituitary there is a gradual loss of melanin pigment from the skin, but experience has shown great individual variation in the degree of fading. In Exp. 1 there was a mean loss of the order of 30% in fish that had been hypophysectomized for 17 weeks. In Exp. 2 the mean loss was only ca. 10% after 13 weeks.

Prolactin therapy, in general, caused a significant increase in the melanin assay of hypophysectomized recipients as compared with untreated or saline-injected hypophysectomized controls. In Exp. 2 highly purified pro-

lactin (C.H.Li) failed to bring about this result, and in Exp. 5 the small increment (9.5%) was not statistically significant ($P=0.1$), but in two other experiments with similar material (Exp. 4 and 7) there were increments of 19.8 and 16.2% respectively. The Schering prolactin (Exp. 3), as anticipated from previous studies, also brought about increased melanization and the Armour preparation (Exp. 1) was highly effective.

Intermedin, whether untreated or alkali-potentiated, consistently failed in these and previous experiments to cause a significant increase in the melanin assay. The results of Exp. 7 are decisive since in this series the intermedin solution was freshly prepared immediately before use, excluding possible deterioration. Similarly, in Exp. 6, there was no significant difference in the melanin assay between freshly prepared untreated and alkali-treated intermedin, and the highest dose of either preparation was no more effective than the lowest.

ACTH caused an increase in the melanin assay of the order of 14%, but on account of high variability, this increase was barely statistically significant ($P=0.05-0.1$).

Combined treatment with purified prolactin and either untreated or alkali-treated intermedin (Exp. 5 and 7) gave melanin assays that were slightly, but not statistically significantly higher than those resulting from prolactin treatment alone. The same was true of the combination of prolactin and ACTH.

Darkening in dopa (Table 3): The increase in melanin pigmentation as a result of incubation of fin samples with dopa may be derived either by comparison with unincubated controls or controls incubated with buffer. In 10 out of 26 experiments the two control readings were essentially alike, and in 5 additional cases the difference between the two reference standards was within reasonable agreement (20–25%) considering the limitations of the method. It would seem justifiable to assume that incubation with buffer had no significant effect on the melanin assay, despite exceptions in which the reading was much lower (9 cases) or much higher (2 cases) than in the unincubated control. The latter is believed to provide a more reliable reference standard since it involves less handling of the sample.

The results of Exp. 1 and 2 show that hypophysectomy markedly diminished or abolished the ability of fin samples to darken when incubated with dopa. Treatment of hypophysectomized fish with prolactin invariably increased this ability. In Exp. 1 and 2 the prolactin-treated hypophysectomized recipients were indistinguishable in this respect from normal controls.

Chronic treatment with intermedin also augments the ability of fin samples to darken *in vitro* with dopa. This was clearly demonstrated in Exp. 7 with alkali-treated MSH at a dose of 8000 units per gm. In Exp. 6, in which untreated and alkali-treated MSH were tested at three parallel dosage levels, there was no difference in the response to the two preparations.

Both evoked a maximum effect at the middle dose (12,000 units per gm.) and the diminished response at 40,000 units per gm. suggests that very high doses may have an inhibitory action.

The combination of prolactin with either untreated or alkali-treated intermedin (Exp. 5 and 7) greatly enhanced the ability to darken in dopa, as compared with either hormone administered alone.

TABLE 3. OXYGEN CONSUMPTION OF FIN SAMPLES INCUBATED WITH DOPA, AND *in vitro* MELANIN SYNTHESIS

Exp. No.[1]	Condition	Treatment	O_2 uptake[2]		Melanin[3]				
						KU		Diff. fr. hypect. control	
			µl./hr./100 mg.	Diff. from hypect. control	Incubation time, hrs.	1	2	1	2
1[4]	Normal	None	7.66	7.33	24	30.9	31.9	16.0	17.5
	Hypect.	None	0.33	—	24	14.9	14.3	—	—
	Hypect.	Prol. (A)	10.66	9.73	24	25.9	31.0	15.0	16.7
2	Normal	None	4.30	2.02	9	29.8	29.5	27.5	34.5
	Hypect.	None	2.28	—	9	2.3	− 5.0	—	—
	Hypect.	Prol. (L)	3.11	0.83	9	30.3	28.0	30.0	33.5
3	Hypect.	None	− 1.13	—	24	1.5	1.9	—	—
	Hypect.	Prol. (S)	3.07	4.20	24	27.9	33.4	26.4	31.5
4	Hypect.	None	− 4.33	—	8	23.5	− 1.0	—	—
	Hypect.	Prol. (L)	9.39	13.72	8	41.0	15.5	17.5	16.5
	Hypect.	α_1-ACTH	15.18	19.51	8	39.5	32.5	16.0	33.5
	Hypect.	Combination	19.25	23.58	8	43.5	5.5	20.0	6.5
5	Hypect.	NaCl	2.28	—	8.5	− 2.0	− 2.0	—	—
	Hypect.	Prol. (L)	3.41	1.13	8.5	11.0	2.0	13.0	4.0
	Hypect.	MSH (1)	1.14	− 1.14	8.5	3.0	5.0	5.0	7.0
	Hypect.	Combination	11.38	9.10	8.5	27.0	18.0	29.0	20.0
7	Hypect.	NaCl	—	—	5.5	6.0	− 1.0	—	—
	Hypect.	Prol. (L)	4.08	—	5.5	29.0	27.0	23.0	28.0
	Hypect.	MSH (3)-OH	6.24	—	5.5	32.0	22.0	26.0	23.0
	Hypect.	Combination	—	—	5.5	70.0	74.0	64.0	75.0
								Diff. fr lowest dose	
6	Hypect.	MSH (1):							
	Hypect.	4,000 u./gm.	—	—	5	16.0	26.0	—	—
	Hypect.	12,000 u./gm.	—	—	5	72.0	44.0	56.0	18.0
	Hypect.	40,000 u./gm.	—	—	5	27.0	10.0	11.0	−16.0
	Hypect.	MSH (2)-OH:							
	Hypect.	4,000 u./gm.	—	—	5	2.0	24.0	—	—
	Hypect.	12,000 u./gm.	—	—	5	45.0	31.0	43.0	7.0
	Hypect.	40,000 u./gm.	—	—	5	22.0	20.0	20.0	− 4.0

[1] See Table 1 for further information.
[2] Calculated from total uptake after 5 hours, except for Exp. 5 and 7 where calculated from total uptake after 4 hours; results corrected for uptake of control samples incubated with buffer alone.
[3] Melanin: 1 = difference between sample incubated with dopa and unincubated control, 2 = difference between sample incubated with dopa and sample incubated with buffer.
[4] Data represents mean of two separate determinations.

ACTH had an effect that was similar to that of intermedin: the ability to darken in dopa was increased to the same extent as by prolactin (Exp. 4). However, no summative or synergic action was observed when the two preparations were combined, in contrast to similar experiments with intermedin.

Oxygen uptake (Table 3): Even with more active preparations the O_2 consumption was low. The hourly uptakes for two typical experiments are shown graphically in Figures 1 and 2. The method precluded statistical treatment since it was necessary to pool the fin samples for each experi-

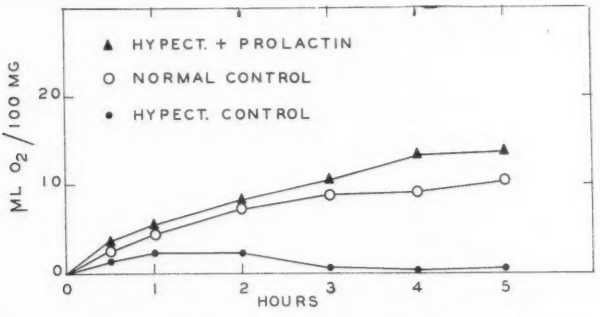

FIG. 1. Exp. 1. Oxygen consumption of fin tissue incubated with dopa. Each point represents the mean of two separate determinations. Liquid phase: 1.5 ml. M/10 phosphate buffer, pH 6.8, containing L-dopa at a concentration of 0.5 mg./ml; 27 mg. of fin tissue, dry weight. Temperature 38° C.

mental group, but the significance of the data is enhanced by the evident close correlation between O_2 uptake and the ability to darken in dopa (Fig. 3).

In Exp. 1 it was found that hypophysectomy results in a decreased ability of the fin samples to utilize dopa, by either criteria. The results of Exp. 2 were less striking.

Treatment of hypophysectomized fish with purified prolactin (C.H.Li) had little effect in Exp. 2 and 5, but in Exp. 4 there was a marked increase in the O_2 uptake. Both the Armour and Schering prolactin preparations were effective (Exp. 1 and 3).

In respect to intermedin, two time consuming experiments failed to give

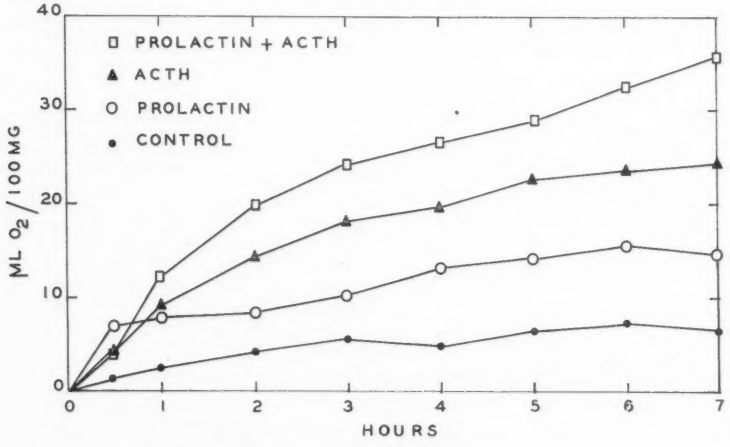

FIG. 2. Exp. 4. Oxygen consumption of fin tissue incubated with dopa. Liquid phase as for Exp. 1, but 30 mg. of fin tissue, dry weight.

decisive results for different reasons. In Exp. 5 it is believed that the prefrozen MSH solutions deteriorated since negative results were obtained by all criteria: melanocyte proliferation, darkening in dopa and increased O_2 consumption. Nevertheless, in the same experiment, the action of this material was potentiated by prolactin and there was no evidence of deterioration during storage when the intermedin was prepared in combination with

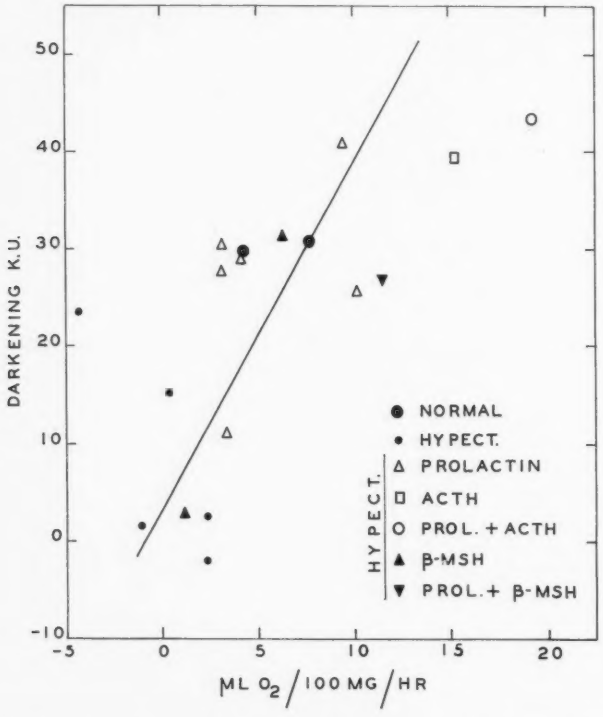

FIG. 3. The significant positive correlation ($r=0.68$) between oxygen consumption and degree of final darkening of fin samples incubated in dopa. The degree of darkening is derived by subtraction from the unincubated controls (Table 3). The mean rate of O_2 uptake is calculated from the total uptake during the first 4 or 5 hours (Table 3).

this protein. In Exp. 7, which was successful in every other respect, the respirometer containing the control fin sample failed leaving in doubt the significance of the apparent rise in O_2 consumption. It would be reasonable to assume that the respiration of the control sample would have been low, as in Exp. 5, and if the data are taken in conjunction with the increased ability of the sample to darken in dopa there is every reason to believe that there was a stimulation of the enzyme system.

ACTH had a striking effect on O_2 consumption (Exp. 4); an effect that

was greater than might have been anticipated from the degree of darkening in dopa (Fig. 3). As with intermedin, the response to the combination of prolactin and ACTH was not significantly greater than with ACTH alone.

DISCUSSION

The overall results of the experiments are summarized in Table 4. In respect to *in vivo* effects, present findings confirm the results of our previous investigations (1) and enlarge our understanding of certain aspects of the problem. Alkali-treated intermedin is approximately ten times more active than untreated material (Exp. 6). In the earlier experiments there was a

TABLE 4. SUMMARY OF RESULTS

Nature of treatment	*In vivo* effects		*In vitro*, incubation with dopa	
	Melanophores	Melanin	Darkening	O_2 uptake
Hypophysectomy, compared with normal	Partial depigmentation	Decrease	Decrease	Decrease
Replacement therapy after hypophysectomy:				
Prolactin	Restoration of pigment	Increase	Increase	Increase
Intermedin	New melanocytes in proportion to dose	No effect[1]	Increase	(Increase?)[2]
Prolactin+intermedin	Abundant new melanocytes, repigmentation	Increase as with prolactin alone[3]	Greater increase, additive or synergic	Increase greater than with prolactin alone
ACTH	Sparse new melanocytes	No effect[1]	Increase	Marked increase
Prolactin+ACTH	Abundant new melanocytes, repigmentation	Increase as with prolactin alone[3]	Increase as with ACTH alone	Marked increase, as with ACTH alone

[1] A slight increase in melanin pigmentation was not statistically significant.
[2] See text.
[3] The melanin assay with combined treatment was slightly, but not significantly greater than with prolactin alone.

lack of agreement regarding the minimum effective dose of intermedin: ca. 110 MSH units in Series XXIIF and ca. 600 units in Series XXVI. Present findings tend to resolve this discrepancy since correspondence with Dr. Irving I. Geschwind revealed that the intermedin employed in Series XXIIF had been treated with alkali whereas that used in Series XXVI had not. Contrary to previous conclusions, Exp. 6 also indicated that higher doses of alkali-treated intermedin increased the speed with which new melanocytes appeared and the numbers that had developed at the end of the experiment. Increased dosage had little effect on the low response to untreated intermedin.

Previous tests with ACTH gave negative results (1), but in view of the melanogenic action of this hormone in the goldfish (12), a new experiment was undertaken (Exp. 4). Each fish received α_s-ACTH containing ca. 132 MSH units per gram weight per injection, approximately equivalent to the minimum effective dose for alkali-treated intermedin. Correspondingly, there was a minimal stimulation of new melanocyte proliferation in 3 fish

out of 10. An apparent small increase in the melanin assay was not statistically significant. Combination of ACTH with prolactin had the same effect as the combination of intermedin with prolactin. As far as Fundulus is concerned, the *in vivo* action of ACTH on melanin pigmentation can be explained in terms of its inherent MSH activity.

In regard to the *in vitro* investigations, there is a general measure of agreement between the O_2 consumption and the ability of the fin sample to darken when incubated with dopa (Fig. 3). Both sets of data must be taken into account in evaluating the activity of the dopa tyrosinase enzyme system. With two possible exceptions (prolactin in Exp. 2 and 5), there was no evidence for a terminal non-oxidative phase of the melanogenic sequence in this species.

Hypophysectomy leads to a marked decrease in dopa tyrosinase activity (Exp. 1 and 2). The partial depigmentation of such fish can therefore be correlated with a diminished capacity for *in vitro* synthesis of melanin. Melanin is continually being lost and replaced in the melanophores of normal fish (13). Our experiments suggest that, in the absence of the pituitary, the replacement mechanism is defective.

Prolactin tends to restore the activity of the enzyme system, in some experiments more effectively than in others. This *in vitro* stimulation is correlated with an increase in the pigmentation of the living animal. However, experiments with intermedin (Exp. 7) and ACTH (Exp. 4) show that stimulation of the enzyme system is not necessarily associated with increased pigmentation. All three hormones promote an increase in dopa tyrosinase activity, but only prolactin is capable of substantially increasing the melanin assay *in vivo*. It seems probable that the endogenous supply of melanin precursor, which may be deficient after hypophysectomy, is specifically restored by prolactin.

Hormonal stimulation of the enzyme system may be basically different, depending upon the diverse modes of action of the agents employed. In the case of prolactin, which fails to induce the multiplication of new melanocytes, the stimulation must influence the intracellular enzyme content of already existing melanophores. On the other hand, the increase in dopa tyrosinase activity after treatment with intermedin may be correlated with the abundance of newly formed melanocytes, each of which presumably contains an enzyme system capable of forming pigment in the presence of an appropriate substrate. In the absence of an adequate supply of endogenous chromagen, new cells would be able to develop pigment only on a restricted basis, apparently deriving their melanin or melanin substrate from material released from pre-existing melanophores. This hypothesis is supported by the lack of evidence for an overall increase in the total melanin assay. The potentiation of the melanocyte-proliferating action of intermedin by prolactin may therefore be attributed not only to prolactin stimulation of the intracellular enzyme system but also to the concomitant

increase in endogenous chromagen, permitting normal differentiation.

The action of ACTH resembles that of intermedin in that there is a stimulation of the dopa tyrosinase system. However, the increase in O_2 consumption was accompanied by a lesser degree of *in vitro* darkening than might have been anticipated (Fig. 3). This suggests a simultaneous stimulation of some non-tyrosinase oxidative enzyme system. Moreover, in contrast to its action in combination with intermedin, prolactin did not significantly augment the activity of the enzyme system beyond that evoked by ACTH alone. Thus while the *in vivo* effects of ACTH on Fundulus pigmentation are in accordance with the inherent MSH activity of the preparation, the *in vitro* effects on the oxidative enzyme system are more complex. However, the evidence is opposed to any specific role of ACTH in the melanogenesis of this species.

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REFERENCES

1. Pickford, G. E. and B. Kosto: *Endocrinology* **61:** 177. 1957.
2. Lerner, A. B.: *Amer. J. Med.* **29:** 902. 1955.
3. Foster, M.: In "Pigment Cell Biology" (Myron Gordon ed.). Academic Press, N. Y.: 301. 1958.
4. Goodrich, H. B.: *Biol. Bull.* **77:** 184. 1933.
5. Goodrich, H. B., N. D. Josephson, J. P. Trinkaus and J. M. Slate: *Genetics* **29:** 584. 1944.
6. Wallbrunn, H. M.: Ph.D. Diss., Univ. Chicago. 1951.
7. Greenberg, S., N. J. Kopac and M. Gordon: *Ann. N. Y. Acad. Sci.* **67:** 55. 1956.
8. Fitzpatrick, T. B., A. B. Lerner, E. Calkins and W. H. Summerson: *Proc. Soc. Exper. Biol. and Med.* **75:** 394. 1950.
9. Humm, D. G. and J. H. Humm: *J. Cell. Comp. Physiol.* **52:** 301. 1958.
10. Humm, D. G. and J. H. Humm: In "Pigment Cell Biology" (Myron Gordon ed.). Academic Press, N. Y.: 197. 1958.
11. Foster, M., R. Cook and T. A. Stamas: *J. Exp. Zool.* **132:** 1. 1956.
12. Chavin, W.: In "Pigment Cell Biology" (Myron Gordon ed.). Academic Press, N. Y.: 63. 1958.
13. Thibault, C. and O. Thibault: *Année Biol.* **51:** 161. 1947.

FACTORS AFFECTING THE THYROID GLAND UPTAKE OF I^{131} OF THE *RANA CATESBEIANA* TADPOLE[1]

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ABSTRACT

Various factors affecting thyroid gland uptakes of I^{131} of the tadpole have been studied. The species of Rana catesbeiana was used since they were widely available the year around in the larval form. All observations, except for one study, were made on non-metamorphosing tadpoles fasted 7 to 14 days. Temperature had a marked effect on the uptake and must be critically controlled to compare responses of these poikilothermic animals. The responses varied during the months of the year being greatest in April and least in October, November and December.

When single injections of TSH and I^{131} were given the greatest difference in the mean uptake between the stimulated and unstimulated tadpoles was observed when TSH was given 68 hours and I^{131} 24 hours before sacrifice. Twenty-five μg. triiodothyronine placed in the tadpole water bath of approximately 1200 ml. suppressed the 24 hour uptake by 50%. This effect occurred within 24 hours and lasted at least 96 hours. The response to TSH was not decreased by the triiodothyronine.

THE tadpole has had a long and successful history as a laboratory animal for the study of problems in thyroid physiology. This investigation was undertaken to study some of the factors affecting the radioactive iodine uptake of the thyroid gland of Rana catesbeiana tadpoles, for this species was widely available. We were especially anxious to study this species because of its large size in the non-metamorphosing stage of development and its slow metamorphosis during a period of two years, which makes the tadpole available the year around in this larval form. All observations were made under laboratory conditions and the tadpoles were handled in the same manner within a restricted temperature range. Unless temperature is critically controlled, it is impossible to compare responses of these poikilothermic animals.

METHOD AND MATERIALS

Two hundred and fifty tadpoles arrived weekly from a commercial breeder and batches of approximately 50 were placed in distilled water in 15 by 10 by 2 inch enamel

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pans covered with wire screens. The water was changed daily or every other day and the tadpoles fasted 7 to 14 days before observations were made. We consistently used the tadpole in the same stage of development, that is, without front legs and with hind legs that were shorter than 5 mm. The tadpole weights ranged from 2 to 10 gm. The average weight and length of the tadpoles most commonly used was 3 gm. and 3 inches, respectively, and the weights of most of the tadpoles in each shipment varied only plus or minus 1 gm. During the period of observation, 10 tadpoles were placed in 11 by 7 by 2 inch enamel pans containing distilled water and covered with wire screens. The pans were kept at various temperatures for 24 hours before studies were started and the water was not changed for the remainder of the experimental period. Five mu. U.S.P. thyrotropin was dissolved in a 0.9% sterile solution of saline and a 0.1 cc. volume was given as a single injection intraperitoneally (IP) or submentally (S), while the controls received 0.1 cc. volume of sterile 0.9% saline solution. The response was determined by I^{131} uptake of the thyroid gland. Approximately one μc. of carrier-free I^{131} in 0.1 cc. saline was given IP or S as a single injection, and after various intervals of time the tadpole was decapitated by the guillotine method. The cartilage forming the jaw and oral pharynx was removed from the head with iridectomy scissors as a 1.5 cm. by 1.0 cm. plate. Then, under a dissecting microscope, the hypobranchial region was exposed by removing the integument, subjacent musculature, heart and accompanying vessels. The thyroid glands lie directly on the cartilage as two vascularized areas, one on each side of the urobranchial cartilage. The thyroid glands were immediately exposed by removing the transverse hyoglossus muscles. The lobes first were freed from the urobranchial cartilage with a 22 gauge hypodermic needle and then were gently stripped off the cartilage with watchmaker's forceps. The thyroid glands, estimated to weigh 100–300 μg., were transferred to filter paper in a stainless steel planchette for counting with a thin mica-end window beta sensitive automatic Geiger-Mueller counter. Under constant geometric relations the glands were counted for 2,560 disintegrations and the response was calculated as counts per second (cps). Since background was always less than 0.5 cps, this was not subtracted from the response. D'Angelo (1) described similar studies using the I^{131} and P^{32} uptake of the thyroid gland of Rana clamitans tadpoles.

RESULTS

Figure 1 shows the effect of temperature on the thyroidal I^{131} uptake of the tadpoles treated with TSH or saline. In the animals treated with TSH there was a marked temperature effect with a maximum response at 26° C. At this temperature the uptake was about 25 times greater than at 3° C and approximately 3 times greater than at 22° or 31 to 33° C. Among the saline treated animals the effect of temperature was much smaller; in one series of experiments the uptake at 26° C was only 10 times greater than at 3° while in the other experiment, in which the temperature varied over a smaller range, 18° to 31°, there was no change in the uptake. It was not possible to correlate variations in body weight with the I^{131} uptake of the saline or TSH treated tadpoles.

The seasonal effect on the I^{131} uptake of untreated non-metamorphosing tadpoles fasted for 7–14 days is shown in Figure 2. The tadpoles were kept at 26° $\pm$2° C during each experimental period. Consistently low uptakes were observed in October, November and December. The highest uptakes occurred in April. In this month the mean uptake of 3 groups of 10 tadpoles

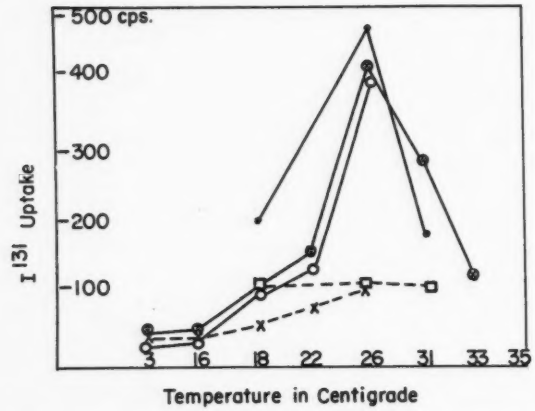

FIG. 1. The effect of ambient temperature on thyroidal uptake of I^{131}. In 3 experiments (solid lines) the tadpoles received 5 mU. of TSH 68 hours before sacrifice. In the control experiments (broken lines) saline was injected instead of TSH. I^{131} was given 24 hours before sacrifice in all experiments. Each point represents the mean value from the thyroid glands of 10 tadpoles.

was 185 cps as compared to 10 cps in 5 such groups in October. It is also noteworthy that the variation in uptakes among groups of tadpoles was greatest in the spring months. A small part of the variability of the results may be due to an inability to control the temperature at 26° C.

Figure 3 shows the effect on the thyroid gland uptakes of varying the time interval between injecting I^{131} and sacrificing the tadpole. The results of the experiment (A) show that the tadpoles treated with saline 68 hours before sacrifice had a gradual increase in the mean uptake as the interval

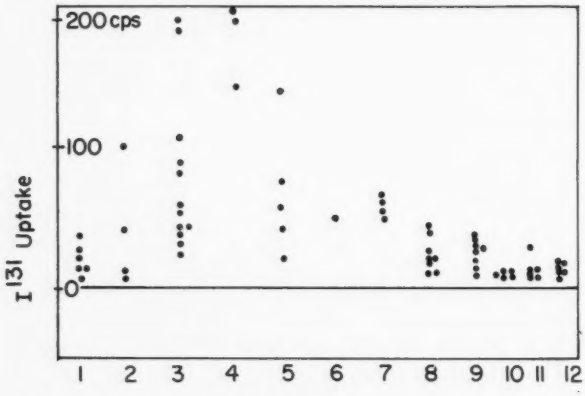

FIG. 2. The 24 hour I^{131} uptakes of untreated non-metamorphosing tadpoles during the months of the year. Each point represents the mean value from the thyroid glands of 10 tadpoles.

between the administration of the I^{131} and sacrificing the tadpole was prolonged. The 2 hour mean uptake was 5 cps and the 24 hour mean uptake 9 cps, i.e., a 1.8 fold increase occurred. In the stimulated groups which received 5 mU. TSH IP 68 hours before sacrifice, the effect of prolonging the interval between I^{131} administration and sacrifice was much greater. The 2 hour mean uptake was 12 cps and the 24 hours mean uptake 152 cps, representing a 13 fold increase over the 2 hour uptake. The greatest difference between stimulated and saline groups was observed when a 24 hour uptake was performed. Experiment (B) of Figure 3, using the same method as above but performed at another time, shows again the 24 hour uptake to

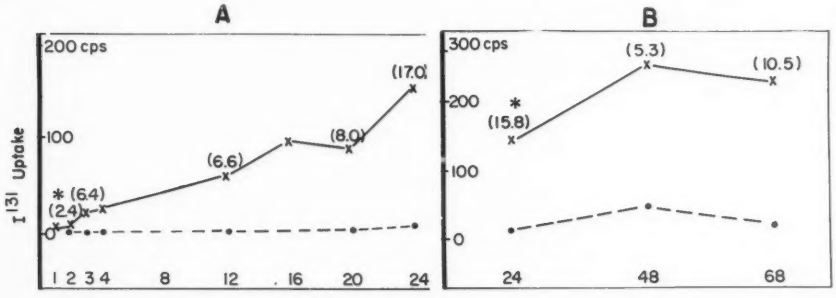

FIG. 3. Effect on thyroidal I^{131} uptake of varying the time between injecting I^{131} and sacrificing the tadpoles. The points joined in the solid line represent the groups of tadpoles which received 5 mU. TSH IP 68 hours before sacrifice. In the control experiments (broken line) saline was injected instead of TSH. Each point represents the mean values from the thyroid glands of 10 tadpoles and above each point is the ratio of uptakes, (stimulated/control).

give a greater difference between the stimulated and saline groups than when the 48 or 68 hour uptakes were used. The saline treated groups had a mean uptake of 47 cps at 48 hours while the 24 and 68 hour mean uptakes were only 11 and 22 cps, respectively.

Figure 4 shows the effect on the 24 hour thyroidal I^{131} uptake of varying the time between injecting 5 mU. TSH and sacrificing the tadpoles. The group stimulated with TSH for 48 hours had a mean 24 hour uptake of 75 cps and at 68 hours 285 cps, i.e., a 3.8 fold increase occurred. We also gave TSH at 72 to 140 hours before sacrifice. After 72 hours there was a gradual decline in the mean uptakes. The seven groups which received saline instead of TSH and I^{131} 24 hours before sacrifice had mean uptakes which ranged from 6 to 13 cps. The mean average uptake of the 68 hour stimulated group was 35 times greater than the mean uptake of the respective saline treated groups.

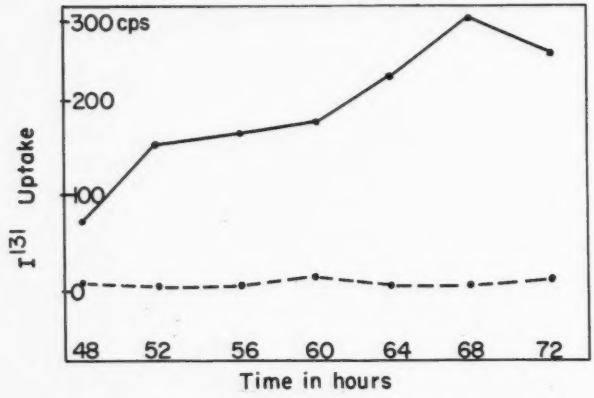

FIG. 4. Effect on 24 hour thyroidal I^{131} uptake of varying the time between injecting 5 MU. TSH IP (solid line) and sacrificing the tadpoles. The control groups (broken line) received saline instead of TSH and I^{131} 24 hours before sacrifice. Each point represents the mean value from the thyroid glands of 10 tadpoles.

TABLE 1. EFFECT ON THE 24 HOUR I^{131} THYROID GLAND UPTAKES BY VARYING THE TIME THE TADPOLES WERE EXPOSED TO 25 μg. OF TRIIODOTHYRONINE IN 1200 ML. BATH WATER

Hours	Untreated (cps)*	Triiodothyronine treated (cps)*
24	117 ± 25	72 ± 11
48	177 ± 32	70 ± 19
72	140 ± 25	53 ± 7
96		70 ± 14

* Mean cps of the thyroid glands of 10 tadpoles ± standard error.

Table 1 shows the effect of the sodium salt of 3,5,3′L-triiodothyronine[4] in suppressing the 24 hour I^{131} uptake of the thyroid gland. Triiodothyronine was dissolved in the 1200 cc. of distilled water in each pan containing 10 tadpoles. In this study, 25 μg. of triiodothyronine was added to the pan and the pans were placed in the incubator at 26° C. Tadpoles were specially obtained with hind legs between 5 and 16 mm. long so that the thyroid gland uptakes would be high. It is noted that the mean cps of the controls were high and the mean averages of the various groups ranged from 117 to 177. This amount of variation is what one observes in the tadpoles of this later stage of metamorphosis. In the treated groups the uptakes were suppressed to approximately 50% of the average control values. This effect occurs within 24 hours and lasts at least 96 hours.

Table 2 shows the effect on the 24 hour I^{131} thyroid gland uptakes by varying the amounts of triiodothyronine given. The triiodothyronine was dissolved in the 1200 ml. tadpole water bath and the tadpoles were sacri-

[4] 3,5,3′L-triiodothyronine tablets, 25 μg., kindly supplied by Smith, Kline & French Laboratories.

TABLE 2. EFFECT ON THE 24 HOUR I^{131} THYROID GLAND UPTAKES OF VARYING THE AMOUNTS OF TRIIODOTHYRONINE IN 1200 ML. OF THE UNSTIMULATED TADPOLES' WATER BATH

0	10**	25	40	50
36* ± 7	19 ± 8	13 ± 3	9 ± 2	died

* Mean cps of the thyroid glands of 10 tadpoles ± standard error.
** μg., triiodothyronine.

TABLE 3. EFFECT ON THE 24 HOUR I^{131} THYROID GLAND UPTAKES OF PLACING 25 μg. OF TRIIODOTHYRONINE IN 1200 ML. OF THE WATER BATH AND GIVING A SINGLE INJECTION OF 5 mu. TSH TO EACH TADPOLE

	No triiodothyronine	With triiodothyronine†
No TSH	240 ± 27*	59 ± 10
5 mu. TSH (IP)‡	643 ± 95	674 ± 67
5 mu. TSH (S)		653 ± 87

* cps—Mean of the thyroid gland of 10 tadpoles ± standard error.
† 25 μg. per pan for 96 hours.
‡ Route of injection.

ficed 96 hours later. Twenty-five μg. of triiodothyronine again suppressed the uptakes to 50% of the control's level. Ten μg. showed a definite effect, while 50 μg. killed the tadpole when used at this temperature for this length of time.

Table 3 shows that the triiodothyronine does depress the I^{131} uptake of the control and does not impair the 72 hour response after a single TSH injection. The route of injection did not affect the response to TSH.

DISCUSSION

In untreated fasted tadpoles environmental temperature and season of the year had an unequivocal effect on the avidity of the thyroid gland for radioactive iodine. The effect of temperature was even greater in fasted tadpoles which had been treated with TSH. In these animals the greatest response to TSH stimulation was at 26° C. Within ±5° C of this temperature there was a three-fold difference.

Fasting is used as a method in attempting to produce a pseudohypophysectomized tadpole, as suggested by D'Angelo (2), but we have not been able to evaluate this procedure in the same group of tadpoles because of the inability to control temperature variation week to week. However, we have observed during the spring that a pseudohypophysectomized state cannot be obtained in this species of tadpole by 14 days of fasting even though the tadpoles are in the non-metamorphosing stage, if one used the I^{131} uptake of thyroid gland as the indicator. The results in Figure 2 show the uptakes to be high despite the tadpoles being in the non-metamorphosing stage and having fasted for 14 days.

When I^{131} is given as a single injection, the 48 hour uptake is greater than

the 24 or 68 hour uptake in the unstimulated tadpole. In order to obtain the greatest difference between the mean uptakes of the TSH stimulated and control groups using single injections of TSH and I^{131}, a systematic study was made by varying the time intervals between giving I^{131} and TSH and sacrificing the tadpoles. The 24 hour I^{131} uptake and the 68 hour TSH stimulation interval gave the greatest differences. By using these intervals we have been able to obtain a dose response curve between 0.5 and 5 mU. using U.S.P. TSH as the reference standard and having assayed TSH levels of unextracted plasma of patients with untreated myxedema and cretinism (3, 4).

During the spring of the year, when the I^{131} uptakes of the unstimulated tadpoles were high even in the non-metamorphosing stage and after fasting, we attempted to suppress the uptake with thyroid hormone. The sodium salt of thyroxin dissolved in alkali injected IP caused a chemical peritonitis and so was not used. Instead, triiodothyronine dissolved in the tadpole bath water was used and was found to suppress the uptakes. We found that 25 μg. of triiodothyronine in 1200 ml. of bath water rather consistently suppressed the uptakes by 50 per cent. This was the optimum amount if a 72 hour or less TSH stimulation period and a 24 hour I^{131} uptake were used for the assay of TSH. At 26° C, although 40 μg. of triiodothyronine does suppress the uptake more than 25 μg., the health of the tadpole is impaired at the end of 3.5 days. The suppressive effect of 25 μg. of triiodothyronine in 1200 ml. of bath water was maximal by 24 hours and lasts for at least 96 hours. In Table 3 the results show that triiodothyronine when given by this method does not impair the TSH response. Also the same response to TSH was obtained with both the intraperitoneal and submental routes of administration.

Comparison of the multiple versus the single injection of TSH has not systematically been studied nor has the release of I^{131} from the thyroid gland. The bioassay of TSH and human serum TSH levels will be reported in a later article using some of the conditions described in this study.

REFERENCES

1. D'Angelo, S. A.: *Proc. Soc. Exper. Biol. and Med.* **92:** 693. 1956.
2. D'Angelo, S. A., A. S. Gordon and H. A. Charipper: *J. Exper. Zool.* **87:** 259. 1941.
3. Bowers, C. Y. and A. Segaloff: *Clin. Res. Proc.* **5:** 109. 1957.
4. Bowers, C. Y., D. Gordon, A. Segaloff: *J. Ped.* **54:** 46. 1959.

METABOLISM OF MONOIODOTYROSINE

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ABSTRACT

The metabolic pathway of monoiodotyrosine in rat kidney and liver was studied by means of paper chromatography using I^{131}-labeled monoiodotyrosine (MIT) and its pyruvic acid (MPP) and acetic acid (MPA) derivatives. In the experiments with liver or kidney slices, MIT was rapidly metabolized into inorganic iodide, MPP, MPA, and several unknown compounds. The conversion of MIT to inorganic iodide was inactivated by grinding or heating the tissues, and by arsenite. On the other hand, MPP itself was more rapidly metabolized than MIT, but MPP was less easily converted to MPA and inorganic iodide. Also, liver and kidney slices showed less activity in converting MPA to other iodinated compounds. It seems unlikely that preliminary deamination of MIT to MPP is important for the subsequent deiodination of MIT or of its metabolites in rat kidney and liver slices.

ROCHE and his co-workers (1) have demonstrated that monoiodotyrosine is readily metabolized in kidney, liver, and thyroid slices of animals, and suggested that this amino acid is deiodinated by a specific deiodinase. Subsequently, Nakano (2) found that I^{131}-labeled monoiodotyrosine is converted into inorganic iodide and several unknown compounds in rat kidney or liver slices, but into inorganic iodide alone in rat thyroid glands. Also, work in this laboratory established that monoiodotyrosine is readily deaminated and deiodinated by L-amino acid oxidase from cobra venom, and it was suggested that deamination may be an important step in the deiodination of this amino acid (3). The present study further defines the relationship of deamination to the deiodination of monoiodotyrosine and identifies certain metabolites of this amino acid in rat kidney or liver.

MATERIALS AND METHODS[2]

Liver and kidney slices and homogenates were prepared from female albino rats (200 gm. ± 10 gm.) maintained on Purina laboratory chow.

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[1] Supported by grants from the John A. Hartford Foundation to the Shadyside Hospital, the Muscular Dystrophy Associations of America, Inc., and the United States Public Health Service.

[2] The phosphate buffer (pH 7.4) used in the experiment was prepared by excluding $CaCl_2$ from the Krebs-Ringer phosphate buffer described by Umbreit *et al.* (Manometric Techniques, Burgess Publishing Co., Minneapolis, Minn., 1948, pg. 194); the nonradioactive monoiodotyrosine was synthesized according to Pitt-Rivers' method (Chemistry and Industry, 21, 1956); the melting points of synthesized compounds were uncorrected.

A. *Slice Experiments*

Tissue slices approximating 200 mg. in weight were suspended in 2.1 ml. of phosphate buffer (pH 7.4) which contained 0.1 ml. of radioactive monoiodotyrosine, 3-iodo-4-hydroxyphenylpyruvic acid, or 3-iodo-4-hydroxyphenylacetic acid in saline with or without arsenite. The incubation vessels were filled with O_2, stoppered and gently agitated for several incubation periods at 37.5° to 38.0°. The contents of the vessels were then brought to a pH of 11.0 to 11.7 by the addition of three drops of 1N-NaOH and homogenized. Aliquots of the homogenate (30 μl.) were then applied to filter paper (Whatman No. 1) and developed chromatographically in collidine-water (100:35.5) in an ammonia atmosphere for 14 to 16 hours at 26°.

B. *Homogenate Experiments*

Homogenates were prepared by grinding the tissues in the phosphate buffer as described by Tong *et al.* (4). Two ml. portions of homogenate were transferred into incubation vessels containing 0.1 ml. of a solution of I^{131}-labeled monoiodotyrosine or I^{131}-labeled 3-iodo-4-hydroxyphenylpyruvic acid in saline. The materials were then incubated for 90 minutes at 37.5° to 38.0°, alkalized and chromatographed as described in the slice experiments.

C. *Snake Venom Experiments*

A stock venom solution was prepared by dissolving 200 mg. of crude cobra venom (Hynson, Westcott, and Dunning) in 1 ml. of the phosphate buffer. One-tenth ml. of the solution was added to 2.1 ml. of Ringer phosphate buffer containing 0.1 ml. of I^{131}-labeled monoiodotyrosine or I^{131}-labeled 3-iodo-4-hydroxyphenylpyruvic acid or I^{131}-labeled 3-iodo-4-hydroxyphenylacetic acid in saline. The samples were incubated for 90 minutes at 37.5° to 38.0°, alkalized and chromatographed as described above.

D. *Radioautography and Counting of Filter Paper Chromatograms*

To determine the location of radioactive compounds on paper chromatograms, a radioautograph of the chromatogram was prepared by placing an x-ray film (Eastman Kodak Co.) directly in contact with the chromatogram for one to two days. Bands of concentrated radioactivity identified by radioautography were cut from the paper. These bands and the intervening areas were counted directly under a mica-window geiger tube without filtration of beta rays.

E. *Identification of Metabolites*

In the identification of metabolites, approximately 80 gamma of non-radioactive authentic compounds (3-iodo-4-hydroxyphenylacetic acid, 3-iodo-4-hydroxyphenylpyruvic acid, thyroxine, monoiodotyrosine, and inorganic iodide) in 0.05 N-NaOH-3% sodium thiosulfate solution were added to alkalized samples obtained after incubation, applied to the filter paper and chromatographed as described. The location of the authentic compounds on the chromatograph was determined by spraying the paper with various color reagents (3).

To complete the identification of metabolites of monoiodotyrosine each band having the same Rf as the authentic sample was cut from the paper and eluted for 24 hours with 50% ethanol alkalized with a few drops of 2N-NH and OH. After evaporation *in vacuo* at room temperature, each residue was identified by cocrystallization with the authentic sample.

F. *Preparation of Iodinated Compounds*

1. *I^{131}-Labeled monoiodotyrosine.* I^{131}-Labeled monoiodotyrosine was prepared by the iodination of L-tyrosine with a mixture of I^{131} (about 3 to 6 mc.) and inorganic

iodine I_2^{127} as previously described (3). The specific activity was about 10 to 20 μc./μg.

2. *I^{131}-Labeled 3-iodo-4-hydroxyphenylpyruvic acid.* I^{131}-Labeled monoiodotyrosine (averaging about 1.25 mc. per 62 μg.) obtained by the above method was dissolved in the phosphate buffer (2 ml.) and incubated with 20 mg. of crude cobra venom for 90 minutes. At the end of this procedure the solution was acidified with 1 N HCl and extracted four times with ether. The pooled ether extract was evaporated to dryness under diminished pressure at room temperature. The residue was dissolved in 50% ethanol placed on the filter paper, and run chromatographically in dioxane-n-butylalcohol-2N NH_4OH (1:4:2) for 12 hours at 26° C. After radioautography, the paper strip corresponding to the pyruvic acid was cut off, eluted with 50% ethanol for 15 hours and evaporated *in vacuo*. The specific activity was approximately 18 μc./μg. just before use.
3. *I^{131}-Labeled 3-iodo-4-hydroxyphenylacetic acid.* I^{131}-Labeled monoiodotyrosine (averaging about 700 μc. per 60 μg.) prepared by the above method was incubated for two hours at 37.5° with approximately 600 mg. of rat liver slices in the phosphate buffer (pH 7.4) containing sodium arsenite at 2×10^{-3} M (5 ml.). After incubation, the reaction mixture was adjusted to pH 2.0 with HCl, and extracted three times with n-butylalcohol. To remove the lipids, the pooled butylalcohol extract was vigorously shaken with an equal amount of chloroform and the mixture extracted three times with 2N NH_4OH ($\frac{1}{3}$ volume of the n-butylalcohol extract), as described by Flock and Bollman (5). The pooled ammonia extract was evaporated to dryness under diminished pressure. The residue was dissolved in 50% ethanol, placed on filter paper and run in collidine water (100:35.5) in an atmosphere of NH_3 for 14 hours at 26° C. After radioautography of the paper chromatogram, the band corresponding to 3-iodo-4-hydroxyphenylacetic acid was cut out of the filter paper, eluted with 50% ethanol, and then dried under diminished pressure. The specific activity of this compound was approximately 9 μc. per gamma just before use.
4. *3-Iodo-4-hydroxyphenylacetic acid.* Four hundred and fifty mg. of 4-hydroxyphenylacetic acid in a mixture of methanol (60 ml.) and concentrated ammonia (30 ml.) were slowly iodinated with 6 ml. of 1 N-iodine solution (Fisher Scientific Co.) at 0° to 4°, while being stirred for three hours. After iodination, the mixture was diluted with 10 ml. of water and then concentrated to 25 ml. under diminished pressure at room temperature. To remove the diiodo derivative of hydroxyphenylacetic acid, the concentrated solution was acidified with a few drops of 10% $NaHSO_3$ and then with 2N HCl during cooling at 0° until precipitation ceased. The brown precipitate was filtered off. The filtrate, adjusted to a pH of 6.0 by the addition of 2N NH_4OH, was evaporated to dryness at room temperature under diminished pressure. The residue was washed three times with small amounts of cold water and re-crystallized from hot water. It was dried at 78.5° *in vacuo* over P_2O_5. The yield was 48%; m.p. 183° to 184°. (Found: C, 32.6; H, 3.4; I, 43.4; $C_8H_7O_3I$ H_2O requires C, 32.5; H, 3.1; I, 42.9%).
5. *3-Iodo-4-hydroxyphenylpyruvic acid.* This compound was prepared in three steps.
   a) *Azlactone of alpha-acetylamino-beta (4-acetoxy-3-iodophenyl) acrylic acid.* Six and two-tenths gm. of 4-hydroxy-3-iodobenzaldehyde prepared by the method of Barnes *et al.* (6) was mixed with 3.0 gm. of acetylglycine, 6.7 ml. of acetic anhydride and 1.5 gm. of fused sodium acetate. The mixture was heated for 40 to 45 minutes over a low flame. After cooling for 12 hours the solid was broken with a glass rod, washed with water and filtered. The yield was approximately 74% of theoretical; m.p. 180°–184°. The compound was used in the next step without further purification.

b) *Four-acetoxy-3-iodo-alpha-acetylaminocinnamic acid.* Six gm. of the above azlactone was refluxed with 540 ml. of a mixture of glacial acetic acid and water (4:5) for 20 minutes. The solution was diluted with 350 ml. of water and allowed to cool for four hours. The brown precipitate was filtered off and the yellowish solution concentrated to one fourth the initial volume under diminished pressure. After cooling for four hours, the yellowish-orange colored prisms were collected by filtration, and recrystallized from dilute acetic acid (25%). The yield was 56.5%; m.p. 198°–199°. (Found: C, 39.3; H, 3.4; I, 32.0; N, 3.6; $C_{13}H_{12}O_5NI$ requires C, 40.1; H, 3.1; I, 32.6; N, 3.6%.)

c) *Three-iodo-4-hydroxyphenylpyruvic acid.* Two gm. of the above cinnamic acid were boiled with 80 ml. of 1.5 N HCl for five hours. The small amount of brown solid which appeared after cooling was removed. The solution was refrigerated for several days. The crystals were collected, washed with cold water and recrystallized twice from boiling water. The product was dried over P_2O_5. The yield was approximately 40% of theoretical; m.p. 168°–171° (decomp.). (Found: C, 35.1; H, 2.5; I, 40.6; $C_9H_7O_4I$ requires C, 35.3; H, 2.3; I, 41.5.)

RESULTS

A. *Metabolism of Monoiodotyrosine by Kidney Slices, Liver Slices and Their Homogenates*

When I^{131}-labeled monoiodotyrosine was incubated with rat liver or kidney slices or their homogenates in the presence or absence of added arsenite it was converted in each instance into several iodinated compounds. The location of these metabolites on radioautographs and the ratio of each metabolite to the total radioactivity are shown in Figures 1 and 2. Three of the bands appearing on radioautographs coincided exactly with the bands obtained with added authentic compounds, including KI, 3-iodo-4-hydroxyphenylpyruvic acid (MPP), and 3-iodo-4-hydroxyphenylacetic acid (MPA). To complete the identification of the metabolites, the bands corresponding to MPA and MPP were cut from the papers obtained in the experiments with the slices or homogenates, eluted, and then crystallized two or three times with authentic samples. From the results shown in Table 1a and 1b most of the radioactivity in the paper bands was presumed to result from MPA, MPP and inorganic iodide. In addition to these three known compounds, two or three bands named X_1, X_2 and X_3, were found. The X_3 band obtained after incubation of MIT with liver slices or homogenates appeared very close to the position taken by added L-thyroxine. However, it is apparent, from cocrystallization of the elutant from the X_3 band that it contained little or no thyroxine (Table 1c). In the incubation of MIT with kidney or liver slices for 90 minutes the conversion ratio of MIT to inorganic iodide was strikingly decreased by arsenite, while the conversion ratio of MIT to MPA was variably increased. Homogenates were quite inactive in converting MIT to inorganic iodide but were still capable of forming MPP, MPA and unknown metabolites. Figure 3 shows the rate at which the metabolites were formed from MIT by liver or kidney slices. In both cases more than one-half of the MIT was rapidly converted

into inorganic iodide within 30 minutes, while accumulation of MPA and the unknown compounds began to increase after 20 minutes of incubation. There was no significant accumulation of the other metabolites.

B. *Metabolism of Monoiodotyrosine by Snake Venom*

When crude cobra venom was incubated with I^{131}-labeled monoiodotyrosine in the phosphate buffer for 90 minutes, with or without arsenite, the

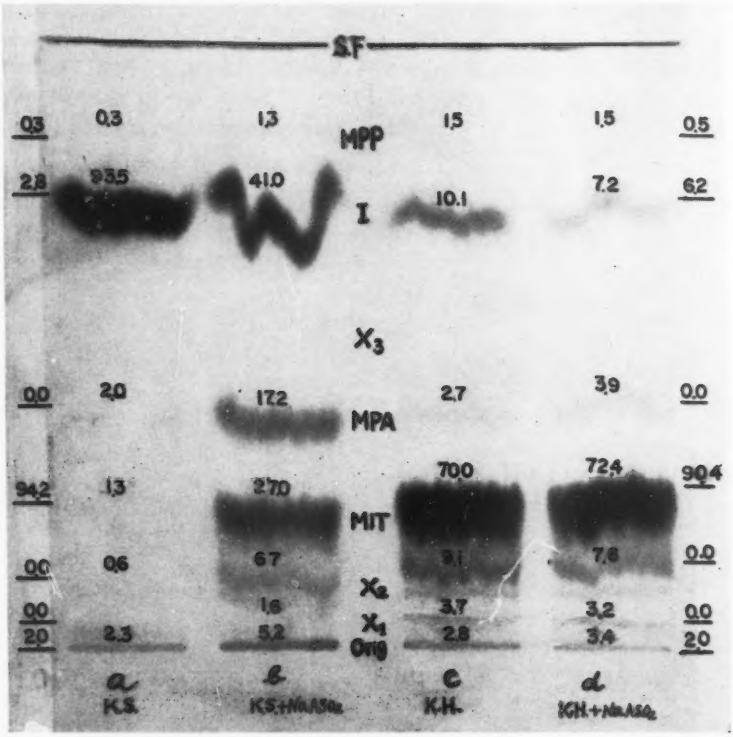


FIG. 1. Radioautographs of paper chromatograms obtained when rat kidney slice or its homogenate had been incubated with I^{131}-labeled MIT (8.4×10^{-6} M for slice experiments; 9.8×10^{-6} M for homogenate experiments) for 90 minutes with or without arsenite (2×10^{-3} M); a) kidney slice, b) kidney slice in the presence of arsenite, c) kidney homogenate, d) kidney homogenate in the presence of arsenite. The number upon the various black bands indicates the percentage of the I^{131} on chromatogram. The number underlined indicates the percentage of the I^{131} on chromatogram in control experiments with heated liver and kidney mixture; left side for use of 8.4×10^{-6} M radioactive MIT, right side for use of 9.8×10^{-6} M radioactive MIT.

S.F. = solvent front.

Orig. = origin.

(Very weak areas of tke dark bands on the original radioautographs do not appear in the photographic reproduction.)

amino acid was almost completely metabolized with the accumulation of MPP and of inorganic iodide in high proportions. However, when the specific activity of radioactive monoiodotyrosine was depressed by the addition of non-radioactive monoiodotyrosine (2×10^{-2}M), the major metabolite of MIT was MPA and not inorganic iodide nor MPP (Fig. 4).

C. *Metabolism of 3-Iodo-4-Hydroxyphenylpyruvic Acid in Kidney Slices, Liver Slices and Snake Venom*

I^{131}-labeled 3-iodo-4-hydroxyphenylpyruvic acid was converted into four or five unknown compounds, MPA and inorganic iodide by kidney slices or liver slices in the presence or absence of added arsenite (Fig. 5). Three of the unknown compounds were found to have the same Rf as the unknown compounds (X_1, X_2 and X_3) formed when monoiodotyrosine was incubated

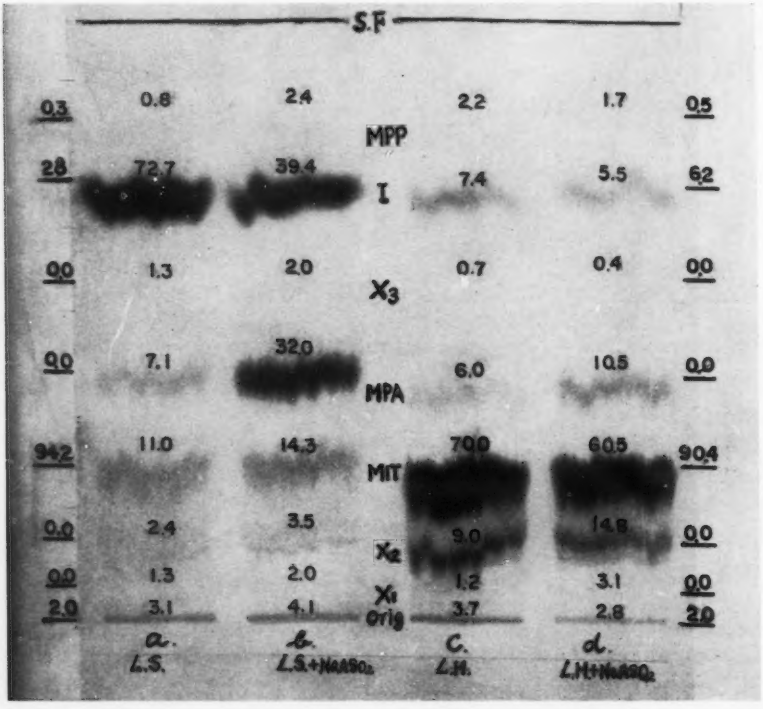


FIG. 2. Radioautographs of paper chromatogram obtained after the incubation of I^{131}-labeled monoiodotyrosine (8.4×10^{-6} M for slice experiments, 9.8×10^{-6} M for homogenate) with liver slice or its homogenate for 90 minutes; a) liver slice, b) liver slice in the presence of arsenite (2×10^{-3} M), c) liver homogenate, d) liver homogenate in the presence of arsenite (2×10^{-3} M). The distribution of iodinated compounds in the heated tissue controls, explained in Figure 1 legend, was rewritten for convenience. See legend for Figure 1 for explanation of numbers and symbols.

TABLE 1. COCRYSTALLIZATION OF RADIOACTIVE METABOLITES OF MIT WITH AUTHENTIC SAMPLE

	(a)	(b)	(c)
	MPA	MPP	X_3
	count/min./μg.	count/min./μg.	count/min./μg.
Original mixture	1.28	2.52	1.46
First cryst.	1.22	2.42	0.05*
Second cryst.	1.22	2.46	0.05*
Third cryst.	1.24	2.45	—

(a) 44 mg. of authentic sample of MPA as carrier were mixed with the radioactive sample obtained from the papers. After the addition of two drops of 10% sodium bisulfite and a small amount of water, the mixture was crystallized three times by heating in a boiling water bath and cooling to 0°–4°. Crystals obtained at each stage were collected, dried to a constant weight over P_2O_5 at 78.5° *in vacuo*, weighed, and counted in a well-type scintillation counter.

(b) 5.3 mg. of authentic samples of MPP were mixed with the radioactive sample. Calculated amounts of 2.4 dinitrophenyl hydrazine and HCl were then added. The hydrazone of MPP obtained by heating and dilution with water was separated and crystallized from 50% ethanol. The crystals obtained at each stage were dried, weighed, and counted, as described above.

(c) 10.2 mg. of L-thyroxine (Smith Kline and French Laboratory) in a slightly alkaline solution were mixed with the radioactive sample obtained from the papers (X_3) and crystallized by the method described by Harington and Barger (*Biochem. J.*, **21**: 169. 1927). The crystals obtained at each stage were dried, weighed, and counted as described above.

* The radioactivity of the crystals was less than two times background.

with preparations of liver and kidney. Unexpectedly, the formation of inorganic iodide and of MPA from MPP in kidney or liver slices was slight, and the major metabolite was compound X_1 in the case of kidney and compound X_3 in the case of liver. Two other compounds were found and named X′ and X″ respectively. The former was located very close to the position taken by added authentic monoiodotyrosine, and the latter in the area between the bands corresponding to MPA and monoiodotyrosine. The conversion of MPP to X′ in kidney or liver slices was markedly enhanced by arsenite. X″ was not present as a visible band on radioautographs obtained in the experiments with liver slices, but it was observed in the experiments with this tissue when arsenite was added and in the kidney experiments in the presence or absence of arsenite. As shown in Table 2 MPP was not metabolized into any other compounds under conditions identical with those employed in the experiments with liver or kidney.

D. *Metabolism of I^{131}-Labeled 3-Iodo-4-Hydroxyphenylacetic Acid by Kidney Slices, Liver Slices and Cobra Venom*

Figure 5 depicts the chromatogram of the metabolites of I^{131}-labeled 3-iodo-4-hydroxyphenylacetic acid obtained by incubation with kidney slices, liver slices, and cobra venom for 90 minutes at 38°. This acid was converted into very small quantities of unknown compounds: X_1 in the kidney and both X_1 and X_3 in the liver. Conversion of the acid to X_1 was found to be inhibited by arsenite. In all instances the formation of inorganic iodide was either slight or entirely absent.

DISCUSSION

A. *Conversion of MIT to Nitrogen-Free Degradation Products*

I^{131}-labeled MIT was rapidly metabolized into inorganic iodide, MPP, MPA and several unknown compounds by rat liver or kidney slices. In radioautographs of all samples obtained before or after incubation of MIT with or without boiling of the tissues, MPP was observed as a very thin band. In addition, there was no significant difference in the yield of MPP obtained with or without prior heating of the tissues. However, it would be premature to conclude that liver and kidney slices are not capable of converting MIT to MPP. Even though MPP might be formed in high proportion from MIT, it should not accumulate in the reaction media. This is supported by the fact that liver or kidney slices converted MPP to another iodinated compound more rapidly than MIT. Since L-amino acid oxidase or transaminases capable of converting aromatic amino acids to the corresponding alpha keto acid have been found in large concentrations in rat

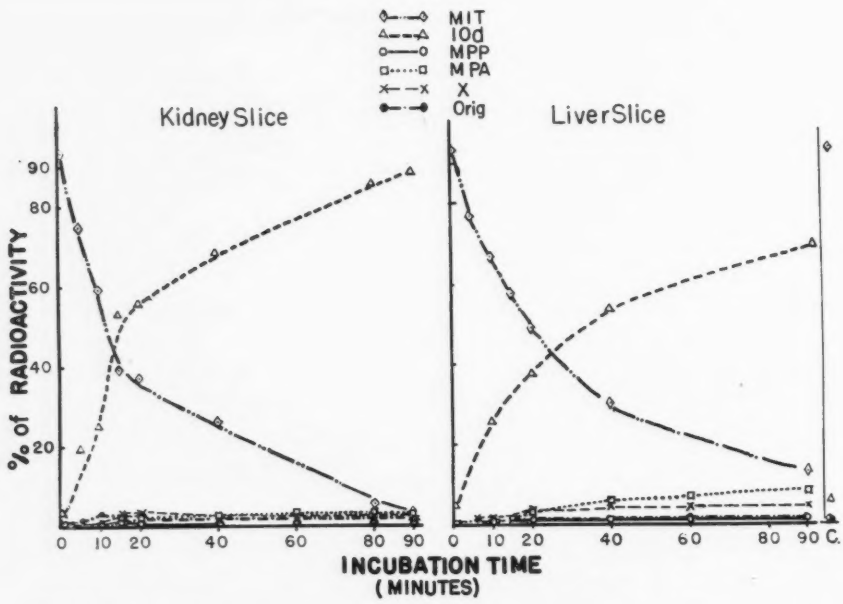


FIG. 3. Degradation rate of monoiodotyrosine in kidney or liver slice. The curves were obtained by plotting the percentage of I^{131} on chromatograms obtained when I^{131}-labeled MIT (9.0×10^{-6} M) had been incubated with kidney slices or liver slices within several time intervals. Zero time sample was obtained by grinding and then alkalizing as soon as the radioactive MIT was incubated with kidney or liver slice. X = total amount of of X_1 and X_2 for kidney slice, total amount of X_1, X_2 and X_3 for liver slice; C = distribution of the iodinated compounds in the heated tissue control.

kidney and liver (4, 7, 8, 9) the formation of MPP from MIT in these tissues would be quite possible.

In contrast to MPP the other nitrogen-free degradation product of MIT, identified as MPA, was less easily metabolized by all enzyme preparations used in our experiments. This acid appears to be a final metabolite of MIT under these experimental conditions. Arsenite, perhaps as an inhibitor of deiodination of MIT, may allow the conversion of MIT to MPA to proceed further. The conversion of iodinated aromatic amino acid to the corresponding acetic acid in rat kidney homogenate has so far been only demonstrated by Tomita *et al.* (10, 11) with regard to the thyroxine and triiodothyronine.

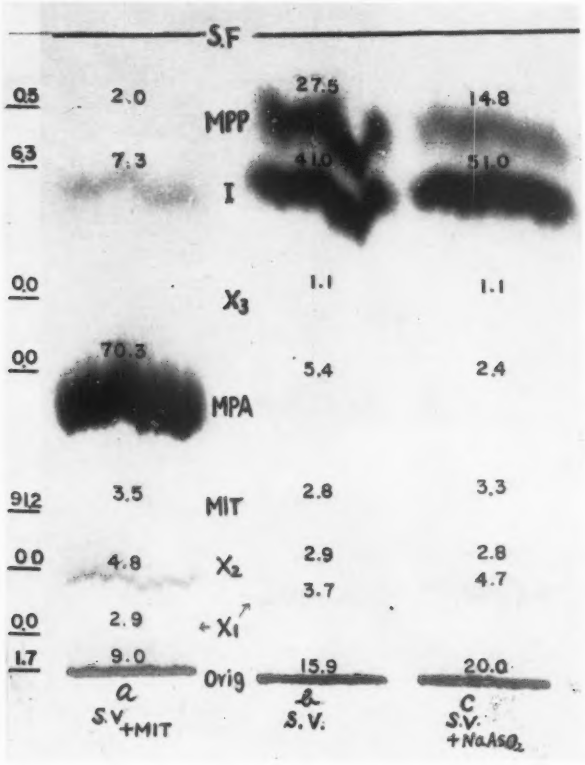


FIG. 4. Radioautographs of paper chromatogram obtained from the incubation experiment of monoiodotyrosine (6.4×10^{-6} M) with cobra venom for 90 minutes: a) cobra venom in the presence of large amounts of non-radioactive monoiodotyrosine (2×10^{-2} M), b) cobra venom, c) cobra venom in the presence of arsenite (2×10^{-3} M). The numbers underlined indicates the percentage of I^{131} on chromatogram in the control experiments (with denatured cobara venom heated for 10 minutes at 100°). See legend for Figure 1 for the explanation of numbers and symbols.

Cobra venom, a crude L-amino acid oxidase preparation, also readily converted MIT to inorganic iodide, MPP, MPA and the unknown compounds if the concentrations of MIT were as low as those employed in the experiments with slices of liver or kidney. However, the results in the cobra venom experiments differed from those with tissue, in that in the former MIT was converted to MPP in high proportion and MPP did accumulate in considerable amounts.

B. *Conversion of MPP to MPA*

Generally alpha keto acids are metabolized in mammalian liver or kidney in accord with two pathways: hydrogenation of the acids to the corresponding hydroxy acids and oxidation of the acids to the corresponding acetic

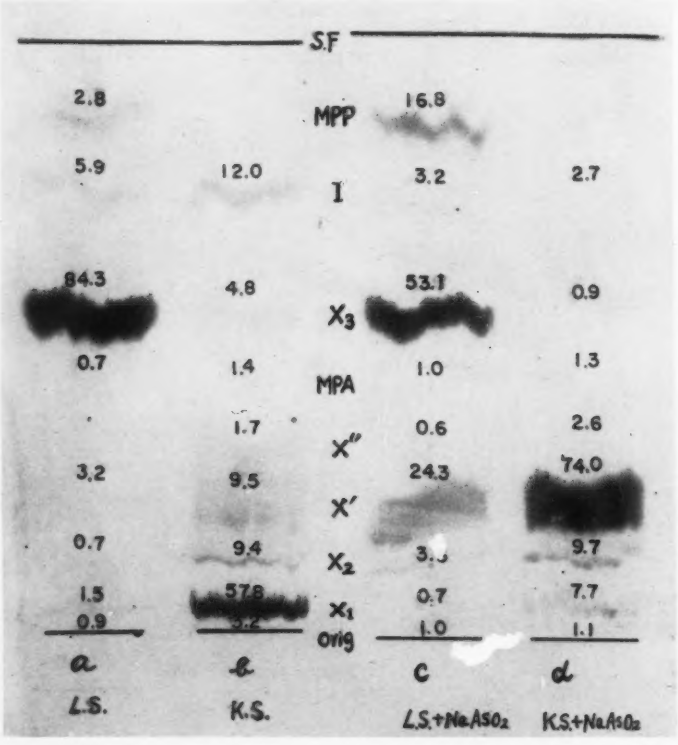


FIG. 5. Radioautographs of paper chromatograms obtained after incubation of liver or kidney slice with I^{131}-labeled MPP (3.8×10^{-6} M) for 90 minutes; a) liver slice, b) kidney slice, c) liver slice in the presence of arsenite (2×10^{-3} M), d) kidney slice in the presence of arsenite (2×10^{-3} M). The distribution of iodinated compounds in heated tissue control is shown in Table 2. See legend for Figure 1 for the explanation of numbers and symbols.

TABLE 2. METABOLISM OF I^{131}-LABELED 3-IODO-4-HYDROXYPHENYLPYRUVIC ACID BY COBRA VENOM

Added 3-iodo-4-hydroxy phenylpyruvic acid (molal concentration)	Enzyme preparation	Added substance (molal concentration)	Per cent of I^{131} on chromatograms found in:								
			Origin	X_1	X_2	X_3	X''	MPA	X_4	1	MPP
3.8×10^{-6} M	Heated tissues*	—	0.2	0	0	0	0	0.1	0	4.2	94.6
3.8×10^{-6} M	Crude cobra venom	—	0.7	0	0	0	0	0.7	0	4.2	93.8
3.8×10^{-6} M	Crude cobra venom	2×10^{-3} M $NaASO_2$	0.1	0	0	0	0	1.0	0	4.3	93.5

* Heated tissues were prepared by heating rat kidney and liver mixture (100 mg. of each) in the phosphate buffer for 10 minutes at 100° and filtered through gauze.

acids. Also, arsenite is known as an inhibitor of oxidative decarboxylation of alpha keto acids (12, 13).

Rat liver or kidney slices manifested very low activity in the conversion of MPP to MPA and arsenite also had no marked effect. On comparison of the conversion ratios of MIT to MPA and those of MPP to MPA by liver or kidney slices, it is apparent that the former occurs to a greater degree than the latter. It seems likely therefore that most of the conversion of MIT to

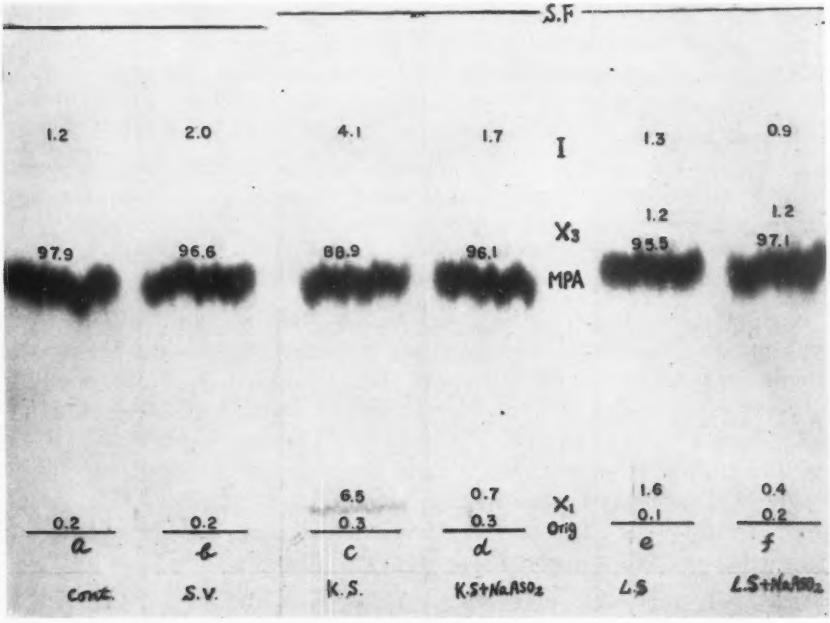
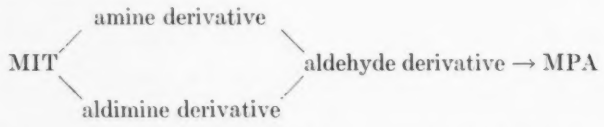


FIG. 6. Radioautographs of paper chromatograms obtained after the incubation of each of enzyme preparations with I^{131}-labeled MPA (4.1×10^{-6} M); a) heated tissue control, b) cobra venom, c) kidney slice, d) kidney slice in the presence of arsenite (2×10^{-3} M), e) liver slice, f) liver slice in the presence of arsenite. See legend for Figure 1 for the explanation of numbers and symbols.

MPA occurs without passing through MPP. We propose the following pathway for this reaction:



Because of possible oxidation of MPP to its aldehyde derivative, the demonstration of an amine or aldimine derivative in the metabolites of MIT is essential for the establishment of this as the actual mechanism involved in the conversion of MIT to MPA. However, we have not established the presence of either an amine or aldimine in these experiments. In previous experiments we have observed however that cobra venom converts MIT to MPP and MPP to inorganic iodide. In the present studies, similar preparations under similar conditions did not convert MPP to any other iodinated compound.

The conversion of MPP to MPA in the case of cobra venom is a chemical rather than an enzymatic reaction, probably involving an oxidative product of MIT such as hydrogen peroxide resulting from L-amine acid oxidase activity, since lower concentrations of MIT result in a lesser conversion to MPA than do high concentrations. This chemical reaction could then be completely stopped by the decomposition of the oxidation product if it is hydrogen peroxide. However, the conversion mechanism of MIT to MPP in liver or kidney slices probably differs from that in cobra venom because the liver and kidney are so rich in catalase which would of course destroy hydrogen peroxide.

C. *Relationship Between Deiodination and Deamination of MIT*

Krebs (12) has found that the activity of L-amino acid oxidase in rat liver or kidney was weakened by grinding tissues and diluting them with saline.

In our experiments with rat liver or kidney the formation of inorganic iodide from MIT was strikingly inhibited by grinding, even though there was no significant difference in the yield of nitrogen-free degradation products of MIT such as MPP and MPA, irrespective of whether MIT was incubated with tissue slices or with their homogenates. This resembles the metabolism of diiodotyrosine in rat kidney and liver, as described by Tong *et al.* (4).

In 1950 Hartmann (14) observed that 3:5-diiodo-4-hydroxyphenylpyruvic acid is easily and spontaneously deiodinated in a phosphate buffer (pH 8.0), and emphasized that deamination of diiodotyrosine is an important preliminary to its deiodination by kidney and liver. However, MPP, the alpha keto acid corresponding to MIT, was very stable in the phosphate buffer used in our experiments (pH 7.4) with boiled liver and kidney

or cobra venom. Also this acid was not easily deiodinated in rat kidney or liver slices.

In our previous experiments with cobra venom, we obtained evidence in support of the possibility that deamination of MIT is directly related to its deiodination. However because of significant differences in the effect of arsenite on the conversion of MIT to inorganic iodide in tissue slices and in cobra venom and in the degradation curves of MIT (Fig. 3 and previous work), it seems more likely that there is no relationship between deamination of MIT and its deiodination in liver or kidney slices. Probably, conversion of MIT to inorganic iodide in rat kidney or liver is mainly performed by one or several enzymes, which is inactivated by grinding or by boiling and which is also inhibited by arsenite. Lissitzky and Bouchilloux (15) have reported that monoiodotyrosine is oxidatively deiodinated by polyphenol oxidase with the formation of dihydroxyphenylalamine, though diiodotyrosine is not deiodinated. However, the enzyme concerned with the deiodination of MIT present in rat liver or kidney is not believed to be polyphenol oxidase, because diiodotyrosine is easily deiodinated by kidney or liver slices as is monoiodotyrosine (4, 16).

REFERENCES

1. Roche, J., R. Michel, O. Michel and S. Lissitzky: *Biochim. Biophys. Acta* **9**: 161. 1952.
2. Nakano, M.: *Endocr. jap.* **4**: 273. 1957.
3. Nakano, M., T. S. Danowski and A. Utsumi: *Endocrinology* **64**: 242. 1959
4. Tong, W., A. Taurog and I. L. Chaikoff: *J. Biol. Chem.* **207**: 59. 1954.
5. Flock, E. V. and J. L. Bollman: *J. Biol. Chem.* **214**: 709. 1955.
6. Barnes, J. H., E. T. Borrows, J. Elks, B. A. Hems and A. G. Long: *J. Chem. Soc.* 2824. 1950.
7. Blanchard, M., D. E. Green, V. Nocito and S. Ratner: *J. Biol. Chem.* **155**: 421. 421. 1944.
8. Knox, W. E. and M. LeMay-Knox: *Biochem. J.* **49**: 686. 1951.
9. Cohen, P. P. and G. L. Hekhuis: *J. Biol. Chem.* **140**: 711. 1941.
10. Albright, E. C., F. C. Larson, K. Tomita and H. A. Lardy: *Endocrinology* **59**: 252. 1956.
11. Tomita, K., H. A. Lardy, F. C. Larson and E. C. Albright: *J. Biol. Chem.* **224**: 387. 1957.
12. Krebs, H. A.: *Biochem. J.* **29**: 1620. 1935.
13. Sumner, J. B. and K. Myrbäck: The enzymes: chemistry and mechanism of action. 1950–52 New York Academic Press.
14. Hartmann, N.: *Z. physiol. Chem.* **285**: 1. 1950.
15. Lissitzky, S. and S. Bouchilloux: *Ciba Foundation Colloquia on Endocrinology* **10**: 135. 1957.
16. Nakano, M. and A. Sugi: *Naibunppi.* **3**: 237. 1956.

GROWTH-PROMOTING EFFECT OF A TRANSPLANTABLE PITUITARY TUMOR IN PARAKEETS[1]

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ABSTRACT

The probable presence of growth hormone in the transplantable parakeet pituitary tumor was indicated by a positive tibia test in hypophysectomized rats. To test for a growth promoting effect in parakeets, 50 birds 2–19 days old were inoculated subcutaneously with the tumor and weighed weekly, 49 nestmates served as controls. Fifteen of the inoculated birds survived 3 months; their average weights and those of the controls were: body weight—51.8 gms., vs 32.2 gms. for the controls; liver 2.55 gms., vs 0.57 gm.; kidneys 0.59 gm., vs 0.30 gm.; heart 0.59 gm., vs 0.43 gm.; brain 1.12 gm., vs 1.13 gm.; pituitary 1.03 mg., vs 0.68 mg.; adrenals 6.0 mg., vs 3.9 mg.; thyroid 11.4 mg., vs 4.4 mg. Inoculated birds were moderately obese, their liver contained larger stores of fat and glycogen than the controls. However, sample cell counts showed that the greater size of the liver was associated with an increase in cell number as well as with a variable accumulation of fat. All inoculated birds showed the tumor-characteristic increase in one component of serum protein.

AMONG the more common tumors of the parakeet, *Melopsittacus undulatus*, are adenomas and carcinomas of the pituitary (1). One of these has been carried as a serial transplant in this laboratory and is now in the 24th passage (2). Many birds with the primary or transplanted tumor become obese, and develop hyperlipemia, hyperglycemia, and polydipsia. All show a marked increase in a plasma protein component, tentatively identified as a globulin (3).

Bioassays of the tumor performed by the Endocrine Laboratories of Madison, Wisconsin were negative for lactogenic hormone; the ACTH activity of the crude extract was less than 0.0005 units per mg. equivalent of tissue. The tibia test for growth hormone showed an increase in epiphyseal width of 38.1 percent over that of the controls when a 500 mg. equivalent of tumor was used per hypophysectomized rat, and of 64.28 percent when 1000 mg. equivalent was injected. It was concluded that a 500 mg. equivalent of tumor tissue (wet weight) contained growth-promoting activity approximately equal to that of 2.0 mg. (dry weight) of growth hormone preparation (Somatotropin).

This paper is a report of experiments designed to obtain evidence for a growth-promoting effect of the pituitary tumor in parakeets.

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MATERIALS AND METHODS

The pituitary tumor tissue was obtained from transplants in the 15th to 17th passage. With needle and stylet, pieces of tumor 2 mm^3 were placed beneath the skin of the back of nestling parakeets. Fifty birds were inoculated, 49 nestmates served as controls. Even with 20–30 breeding pairs, all genetically unrelated, it is difficult to have large numbers of young nestlings available at a given time. For this reason, an average of 12 birds from 2–4 nests was studied simultaneously, the total of 50 inoculated nestlings and 49 nestmate controls represents the sum used in 8 experiments.

The effect of a transplantable parakeet renal adenocarcinoma (11th passage) was determined in a single experiment using 16 nestlings in 5 nests; 8 were inoculated, 8 served as controls.

The age of nestlings at the time of inoculation and of their controls averaged 9.5 days (range 2–19 days). This variation in the age of the birds at the beginning of the experiment was due to the fact that eggs are laid at 2 day intervals. In a nest of 6 young birds the first to hatch is 10 days older than the one from the last egg laid. The young usually number 2 to 6 per nest; alternate birds were inoculated, the others were controls. To diminish the significance of the unavoidable age differences, birds were not killed until 50 and 83–90 days after inoculation; those that died at other times were examined in the same manner as those sacrificed. Birds from the same nest were used for inoculation and control to rule out variations between the offspring of different parents that might be due to differences in parental care.

Nestlings were weighed on the day of inoculation, then twice weekly for the first 5 weeks and once each week thereafter. Before weighing, the birds were separated from the parents for several hours to allow time for the crop to empty. In very young birds a full crop would contribute greatly to the total weight of the bird.

Approximately 1.0 ml. of blood was removed by intracardiac needle puncture at the time of sacrifice using a heparin moistened needle and syringe. The blood was transferred to silicone treated tubes, centrifuged, and the plasma used for paper electrophoresis to determine the protein pattern (3). In a few instances blood sugar levels were obtained using the technique of Reinecke (4). Micro-hematocrit determinations were made with heparinized capillary tubes spun down in an International micro-capillary centrifuge and read with a Spiracrit micro-hematocrit reading device.

After bleeding, the birds were killed by dislocating the cervical vertebrae. The heart, kidneys, liver and brain were weighed at the time of autopsy using a Fisher triple beam balance sensitive to 0.01 gm. The pituitary, thyroids and adrenals were fixed in 10% formalin for several days. After removal from the fixative they were placed on filter paper to absorb excess fluid, then weighed on a Roller-Smith torsion micro balance. The size of the tumor transplants was measured with a millimeter rule. All tissues were fixed in formalin and sectioned for histological examination.

RESULTS

All nestlings, inoculated and control, showed a rapid gain in body weight during the first two weeks (Figs. 1 & 2). This was most pronounced in the youngest birds. The control nestlings, as well as those inoculated with renal adenocarcinoma, reached a stable weight of 25–35 gm. when 4 to 5 weeks old (Fig. 2). In contrast, nestlings having pituitary tumor transplants continued to increase in weight in the second month, leveling off during the third at 45–64 gm. (Fig. 1).

A month after inoculation many birds bearing the pituitary tumor had

become moderately obese. This was characterized by the accumulation of fat depots beneath the skin of the breast and abdomen and in the mesentery. Furthermore, disregarding the fat depots, birds sacrificed 3 months after inoculation, appeared somewhat larger than their nestmate controls. The mean weight of the former was 51.8 gm., of the latter 32.2 gm. By contrast the mean weight of birds sacrified 3 months after inoculation with renal adenocarcinoma was 32.4 gm., that of the controls 32.1 gm. The

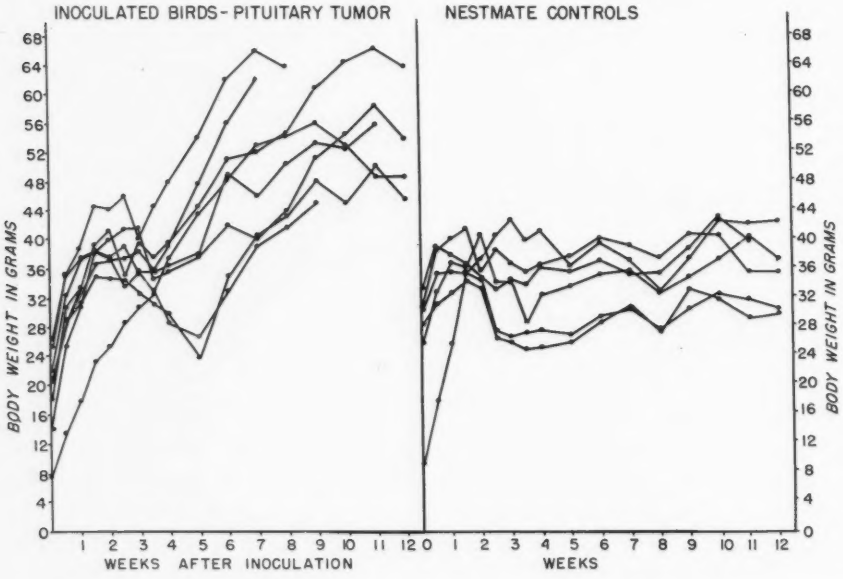

FIG. 1. Effect of pituitary tumor transplant on body weight of nestlings inoculated when 9–13 days old, compared with the weight of nestmate controls 5–17 days old at beginning of experiment. Lines terminating before 12th week indicate death of bird. Data are from one of 8 similar experiments, results of the other experiments matched these very closely.

weight of the pituitary and renal tumor transplants at the time of autopsy ranged from 0.5 to 3.0 gm., with a mean of 1.0 gm.; insufficient to significantly alter the total body weight.

Organ weights of these birds were determined for the liver, kidneys, heart, brain, pituitary, adrenals and thyroids. The parakeets having kidney tumor transplants showed no significant differences from their nestmate controls except for a heavier liver in the former and larger heart in the latter. In birds with pituitary tumor transplants however, all organs except the brain were heavier than those of the controls to a degree that is statistically highly significant (Table 1).

Of the 50 nestlings with pituitary tumor transplants 5 were sacrificed 50

days after inoculation, along with their 6 nestmate controls, 15 were sacrificed 3 months (83–90 days) after inoculation simultaneously with their controls. The most striking effect of the pituitary tumor on visceral weights is observed in the liver. Fat stains showed an increased amount of fat and glycogen in the liver cells as compared with that in the controls, however, the cells were not noticeably enlarged. Cell counts of representative high power fields were not significantly different in the inoculated and control birds, indicating that the greater size of the liver in the former was associated with an increase in cell number, as well as with a variable accumulation of fat.

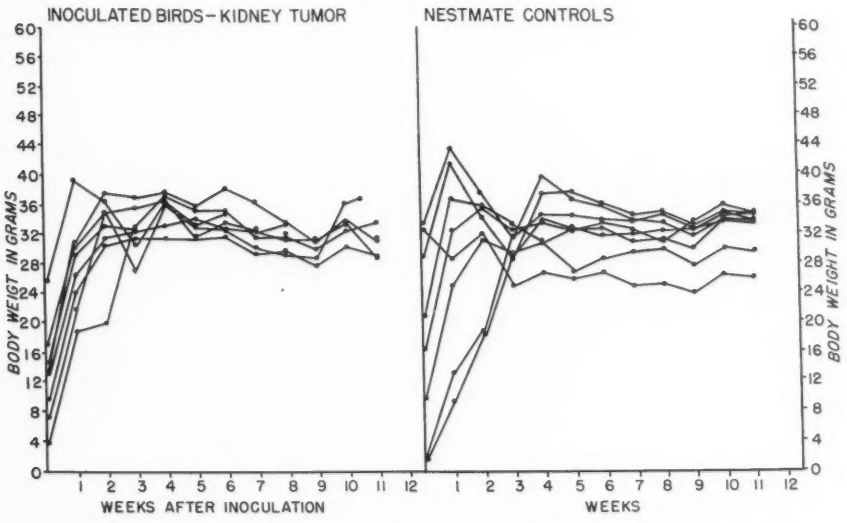

FIG. 2. Effect of renal tumor transplant on body weight of nestlings inoculated when 8–13 days old, compared with growth of nestmate controls 2–19 days old at beginning of experiment.

In the kidneys of inoculated birds there was also a slight increase of intracellular fat, but the nuclear-cytoplasmic ratio was not affected and the greater weight of the organs is attributed to an increase in functional tissue. The greater average cardiac weight of the inoculated birds compared with that of their nestmate controls was not associated with any histological differences. This is not unexpected since in the chicken, and perhaps in most birds, there is little change in size of the myocardial fibers over the entire range of heart growth (5).

In several instances the ovary of an inoculated bird was larger, with more mature follicles, than any observed in control nestmates; similarly, large testes with active spermatogenesis were more frequently found among inoculated birds. However, no quantitative data were collected.

No effect of the pituitary tumor transplant was observed on the growth and size of the brain (Table 1).

The 3 endocrine glands examined were larger in the inoculated than in the control birds. Of particular interest is the increased size of the pituitary, since the changes noted in the adrenals and thyroid may be secondary to an increased activity of the pituitary rather than to the tumor transplant. This possibility is supported by the fact that young adult parakeets inoculated with the pituitary tumor do not exhibit enlargement of the pituitary, nor is there hyperplasia of the thyroid or increased size of the adrenals (2). A definitive histologic study of these glands has not been completed.

The pancreatic islets also await further histologic study and evaluation. Blood sugar levels were determined in only 6 inoculated birds (average,

TABLE 1. MEAN BODY AND ORGAN WEIGHTS OF BIRDS SACRIFICED 3 MONTHS AFTER TUMOR TRANSPLANTATION

	No. of Birds	Body	Liver	Kidneys	Heart	Brain	Pituitary	Adrenals	Thyroid
		gm.	gm.	gm.	gm.	gm.	mg.	mg.	mg.
Birds with Pituitary Tumor Transplants	15	51.8	2.55	0.59	0.59	1.12	1.03	6.0	11.4
Nestmate Controls	22	32.2	0.57	0.30	0.43	1.13	0.68	3.9	4.4
P Value		<.001	<.001	<.001	<.001	n.s.	<.001	<.001	<.001
Birds with Kidney Tumor Transplants	7	32.4	0.80	0.30	0.40	1.09	0.60	5.07	5.07
Nestmate Controls	7	32.1	0.54	0.27	0.50	1.07	0.63	3.80	5.62
P Value		n.s.	<.025	n.s.	<.01	n.s.	n.s.	n.s.	n.s.

98 mg.%) and 7 nestmate controls (average, 85.5 mg.%). The slight elevation of the blood sugar in the inoculated birds may be compared with the marked hyperglycemia found in many adult birds bearing the tumor (2). Weil (6) points out that in growing animals; e.g. puppies and kittens, hyperglycemia usually cannot be produced by growth hormone because of the functional reserve of the pancreas and the greater demand for protein.

Serum electrophoresis was carried out on the blood of 26 inoculated parakeets and 28 nestmate controls. In all inoculated birds there was an elevation of the second component, probably a globulin, similar to that found in adult birds bearing pituitary tumor transplants (3). No elevation of the second component was seen in the serum of nestmate controls. In the inoculated birds this component averaged 54% of the total protein, among the controls it was 28%. Elevation of the second component was absent from the serum of birds with the renal tumor transplant and of their nestmate controls.

Hematocrit determinations gave values of 44.5% (range 23–58) for parakeets with pituitary transplants; 47% (range 34–58) for their nestmate controls, a difference which is regarded as not significant.

DISCUSSION

The increase in body and organ weight that accompanies growth of the pituitary tumor transplants is unique among parakeet tumors studied in this laboratory. It has not been observed to occur with other neoplasms that are carried in serial passage viz., renal adenocarcinoma, squamous cell carcinoma, rhabdomyosarcoma and 3 fibrosarcomas. In view of the positive result of the hypophysectomized rat tibia test with an extract of the tumor, it is believed probable that the changes in nestling parakeets may likewise be attributed to a growth hormone secreted by the tumor. Unfortunately, it has not yet been possible to hypophysectomize parakeets, all birds have died during or immediately after operation.

Adult parakeets inoculated with pituitary tumor show no consistent changes in the pituitary, thyroid or adrenals. The pituitary of inoculated nestlings, however, is enlarged and it appears probable that the adrenal and thyroid hyperplasia is secondary to increased activity of the host's pituitary, rather than to a direct effect of the tumor transplant. Support for this is found in the absence of significant changes in the thyroids and adrenals of birds with spontaneous pituitary tumors, of which nearly 200 have been examined. On the other hand an increase in body weight, enlarged liver and marked hyperglycemia are characteristic of the spontaneous tumor (1) as well as of the transplant growing in adult birds (2). Even after the nestling data have been corrected for the differences in mean body weight of the control and inoculated birds, the weights of the thyroid and liver of the latter show a significant relative increase. As noted in the preceding section, the augmented weight of the liver was associated with an increase in cell number.

Whether the hormone secreted by the pituitary tumor is a growth hormone similar to that of mammals remains to be determined. Very little is known about an avian growth hormone. As recently as 1954 Sturkie (7) stated that the presence of growth hormone in the pituitaries of birds had not been demonstrated. In a personal communication, Dr. C. H. Li (8) wrote that chicken pituitaries assayed in his laboratory were found to contain very little growth hormone activity by the tibia test. A total dose of 0.5 mg. whole chicken pituitary tissue did not cause any increase of tibia width in hypophysectomized female rats. The same total dose of beef pituitary tissue has very marked effect. The effect of hypophysectomy on growth, studied in chickens (9, 10) and pigeons (11, 12) has been equivocal.

REFERENCES

1. Schlumberger, H. G.: *Canc. Res.* **14**: 237. 1954.
2. Schlumberger, H. G.: *Canc. Res.* **16**: 149. 1956.
3. Schlumberger, H. G. and R. L. Wall: *Proc. Soc. Exper. Biol. and Med.* **96**: 43. 1957.
4. Reinecke, A. M.: *J. Biol. Chem.* **143**: 351. 1942.

5. Black-Schaffer, B. and M. E. Turner: *Am. J. Path.* **34**: 745. 1958.
6. Weil, R.: *AMA Arch. Int. Med.* **95**: 739. 1955.
7. Sturkie, P. D.: Avian Physiology p. 312, Comstock Publ. Assoc., Ithaca, N. Y., 1954.
8. Li, C. H., Hormone Research Laboratory, Univ. of Calif., Berkeley, California, June 9, 1959.
9. Nalbandov, A. and L. E. Card: *J. Exper. Zool.* **94**: 387. 1943.
10. Rothchild, I.: *Endocrinology* **43**: 298. 1948.
11. Schooley, J. P.: *Endocrinology* **25**: 372. 1939.
12. Schooley, J. P., O. Riddle and R. W. Bates: *Am. J. Anat.* **69**: 123. 1941.

THE RELATIONSHIP OF THE PITUICYTE TO THE POSTERIOR LOBE HORMONES[1]

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ABSTRACT

When adult male white rats were dehydrated either by the withholding of water or by the administration of a 2.5% sodium chloride solution an increase in mitotic activity of pituicytes was noted. Cell counts indicated that the number of mitotic figures increased until a peak was reached (at four days for the NaCl treated animals and six days for the water-deprived animals) after which the mitotic activity gradually returned to normal even though dehydration was continued.

If these results are combined with the numerous observations of pituicyte changes under a variety of conditions in which posterior pituitary factors are known to be released, it would appear that a more than coincidental relationship exists between the activity of the pituicyte and the release mechanism. Further, a comparison of the morphological and pharmacological events which obtain during dehydration leads to the conclusion that the function of the pituicyte is broader than one of support.

THAT the cellular elements of the neurohypophysis are in no way responsible for the synthesis of the posterior lobe hormones is by now a well documented and accepted fact (1, 2). However, the acceptance of the hypothalamic neurosecretory concept for the origin of the posterior lobe hormones by transferring the site of synthesis of the hormones from the posterior lobe of the pituitary gland to the hypothalamus left many questions unanswered with regard to the function of the pituicytes. Such questions include the presence and mobility of cytoplasmic granules as reported by Gersh (3) and Gersh and Brooks (4) and the apparent hypertrophy of nuclei and the apparent increased mitotic activity of the pituicytes as reported by Selye and Hall (5), Stutinsky (6, 7), Rennels and Drager (8), Ortmann (9), and Billenstien and Leveque (10) among others.

Although, as previously stated, it was felt that the pituicytes played no role in hormone synthesis, it was nevertheless apparent that the reported pituicyte modifications, such as hypertrophy of the nucleus and increased mitotic activity, occurred at times when the posterior lobe hormones were mobilized. Therefore, since the question of hormone release from the neurohypophyseal tissue still remained open, we thought it possible that the pituicyte might play a role in this release.

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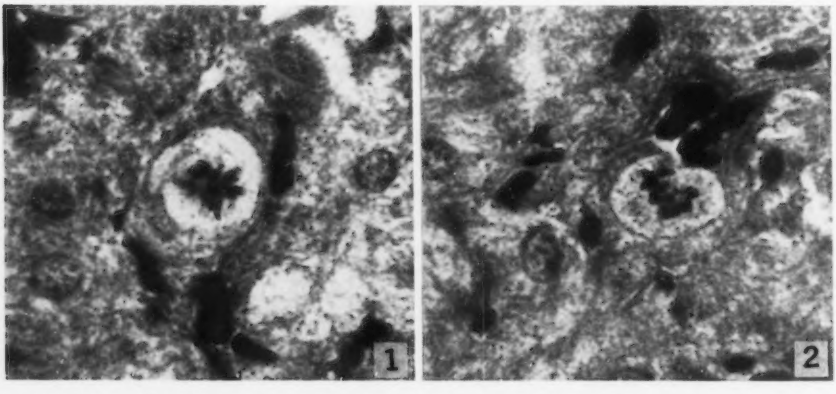
Mitotic activity which accompanies dehydration was chosen as a means of investigation for it seemed to be the most constant observation and also could be determined more easily than measurement of nuclear hypertrophy. However, before definite correlations could be made, it was deemed necessary to establish the following facts: First, that any increase in mitotic activity during dehydration was indeed a real one. Second, that this increase in mitotic activity was not due primarily to any specific effect of of NaCl. Third, that the increased mitotic activity was limited to the tissue under study. As a consequence, it was necessary to include a consideration of these facts in the following experiments.

MATERIALS AND METHODS

Ninety adult male white rats of the Holzman strain ranging in weight from 150 to 240 gm. were divided into five different groups. One group served as untreated controls. In a second control group, 0.1 gm. of colchicine per 100 gm. of body weight was administered subcutaneously six hours prior to sacrifice. A third group was dehydrated by the administration of 2.5% sodium chloride as drinking fluid. A fourth group was dehydrated by the administration of 2.5% NaCl and also given 0.1 mg. of colchicine subcutaneously. A fifth group was dehydrated by the withholding of water. Representative animals from each group were sacrificed at 3:00 P.M. each day, starting with the twenty-fourth hour of dehydration.

The brain, pituitary gland, adrenals, salivary gland, and thyroid gland were removed from each animal and fixed in Zenker-formol. The tissues were embedded in paraffin and cut serially at 7 u. The sections were mounted and then stained with Foot's modification of the Masson Trichrome Strain.

Total cell counts were made of twenty oil immersion fields taken at random from all of the tissues removed from each animal. The fields were projected through the straight tube of an A/O Spencer Trinocular Microscope on to the ground glass of a Reichert Viewscope to which a piece of 0.003″ acetate mattex had been affixed with rubber cement. The nuclei were circled in pencil on the matted surface of the acetate. The acetate was then removed and the circles representing the nuclei were counted. Only



FIGS. 1 and 2. Pituicytes in metaphase during dehydration. Zenker-formol fixed, Foot's modification of Masson Trichrome stain. ×950.

those cells which were in metaphase were utilized in the mitotic counts (Figs. 1 and 2). The counts from individual animals in each group were so close that averages were calculated and expressed as the number of metaphase figures per 1000 cells.

RESULTS

A. Neurohypophysis

1. *Control Group.* Although an occasional pituicyte can be seen undergoing mitotic division in the control animals, the rate of mitosis is so low as to be considered zero for the purposes of these experiments.

2. *Colchicine Control Group.* The administration of 0.1 mg./kg. of colchicine to the animals six hours prior to sacrifice established a baseline for mitotic activity at a level close to 1 per 1000 cells (Fig. 3). The concentration of colchicine used had been arrived at as being optimum through prior tests with solutions of a variety of concentrations. Since all animals, whether injected with colchicine or not, were sacrificed at 3:00 p.m., it is felt that the possibility of any part of the increase in mitotic activity being attributed to a cyclic phenomenon is minimized.

3. *Dehydrated 2.5% NaCl.* With the onset of dehydration, the mitotic

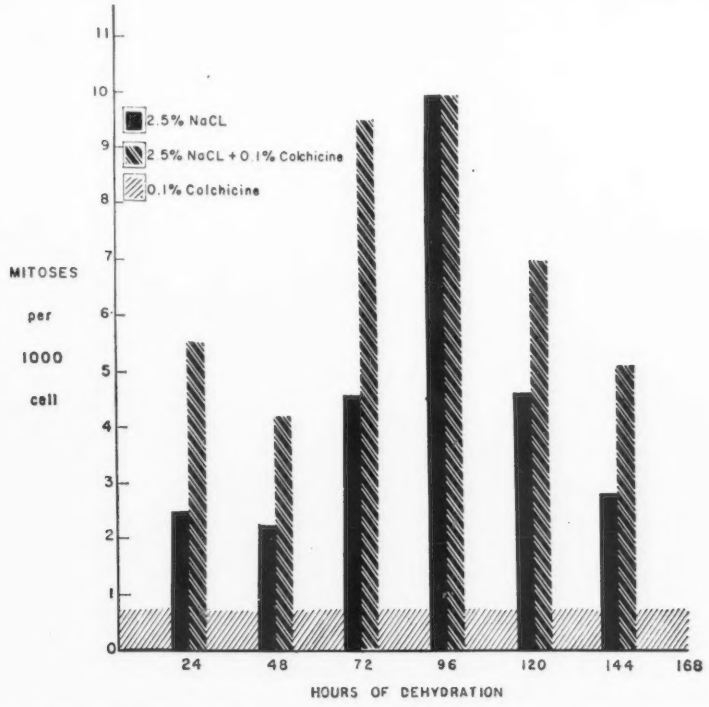


FIG. 3. Number of mitotic figures per 1000 pituicytes following administration of colchicine alone, 2.5% NaCl alone, and 2.5% NaCl plus colchicine. Colchicine = 0.1 mg./kg.

activity increases gradually from the first day until a peak is reached at ninety-six hours (Fig. 3). Following this peak, the mitotic activity gradually decreases until the normal level is regained between the eighth and tenth day.

4. *NaCl Dehydrated Animals Administered Colchicine.* The pattern observed in these animals is essentially that seen in animals from group 3 above. However, in contrast to the latter, the number of mitotic figures

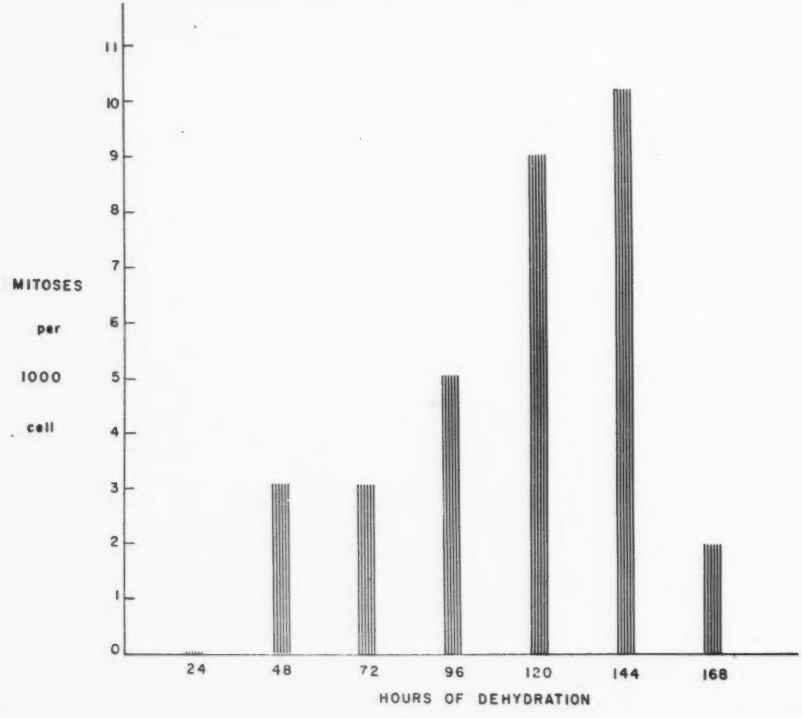


FIG. 4. Number of mitotic figures per 1000 pituicytes following withholding of water.

counted was roughly double that of the animals given NaCl alone. The only exception was the counts made on the 96th hour (Fig. 3). Although no complete explanation can be advanced for the fact that the mitotic count of the two groups are equal at this time, it might be that the activity attained is close to maximum for the tissue.

5. *Dehydration By With Withholding Water.* The over-all effect of dehydration by the withholding of water is the same as that obtained from the administration of NaCl. However, it is interesting to note that in the case of the animals deprived of water, the increase in mitosis is more gradual and a maximum is not reached until the 144th hour (Fig. 4). This would tend to support the idea, already formulated from other observations, that

the administration of a 2.5% NaCl solution is quicker and more effective in producing dehydration.

B. Nervous and Glandular Tissues

Representative areas of the brain, the pars distalis of the pituitary, the adrenal gland, the thyroid gland, and the salivary gland were also studied with regard to the effect of dehydration on mitotic activity. In none of these tissues was an increase noted except for the pars distalis of the pituitary gland. The effects on the latter are now under study and will be the subject of a future communication.

DISCUSSION

The results of these experiments would seem to leave little doubt that the increase in mitotic activity observed in the neurohypophysis of the rat during the process of dehydration is indeed a real one. Further, it can be seen that dehydration had no apparent effect on mitotic activity in the other nervous or glandular tissues studied. These facts combined with the numerous observations of nuclear or cytoplasmic changes in the pituicytes under experimental conditions (3, 4, 5, 6, 7, 8, 9, 10) in which posterior lobe hormones are known to be released would strongly suggest that, from a morphological point of view, a relationship exists between the pituicytes and the hormones and that this relationship is more that coincidental.

Nevertheless, it could be argued that the presence of an observable increase in mitotic activity in animals which were dehydrated by the withholding of water is no proof that sodium chloride is not the agent responsible for the phenonemon. However, the gradual return to normal levels of mitoses even though dehydration is continued would tend to indicate that sodium chloride itself is not the prime factor. If it were, it would appear more likely that mitotic figures would either continue to increase in number or reach a plateau which would then be maintained until the termination of the experiments. The absence of an increase in mitotic activity in the other glandular or nervous tissues studied, except for the pars distalis, also tend to mitigate against an effect of sodium chloride.

Since the morphological events indicated a relationship between the pituicytes and the posterior lobe factors it was of interest to determine whether this relationship could be further clarified by a study of the hormone content of the pars nervosa as dehydration progressed. Recently, Dicker and Nunn (11) reported that, with dehydration, the posterior pituitary content of vasopressin increases for the first forty-eight hours and then gradually decreases until but little activity can be detected in extracts made of the gland beyond the 120th hour. Since the presence and mobility of cytoplasmic granules can be demonstrated in the pituicyte (3, 4), these granules may represent a substance/or substances which causes the release of or the separation of the hormones from a "carrier substance". If this

concept is accepted, the gradual rise followed by a fall in hormone content of the neurohypophysis following dehydration could be explained on the basis of the fact that initially the number of pituicytes is insufficient to produce the amount of substance necessary to cause a massive release or separation. As a consequence, the cells are stimulated to divide until such time as a sufficient number exists which would be great enough to meet any requirement. This hypothesis would seem to receive support from the observations of Rennels (personal communication) who found that in rats which were subjected to repeated bouts of dehydration followed by rehydration most, if not all, of the increase in the mitotic activity of the pituicyte occurs during the first period of dehydration.

Therefore, although the status of the pituicyte is unchanged with regard to posterior lobe hormone synthesis, the correlation between cell morphology and the pharmacological activity of the tissue tends to implicate the pituicyte in either the release mechanism or the separation of the hormones from a "carrier substance". In view of this conclusion, we would propose that the function of the pituicyte is broader than that of mere support and that the earlier diagram of Leveque and Scharrer (1) be modified accordingly as shown in Fig. 5.

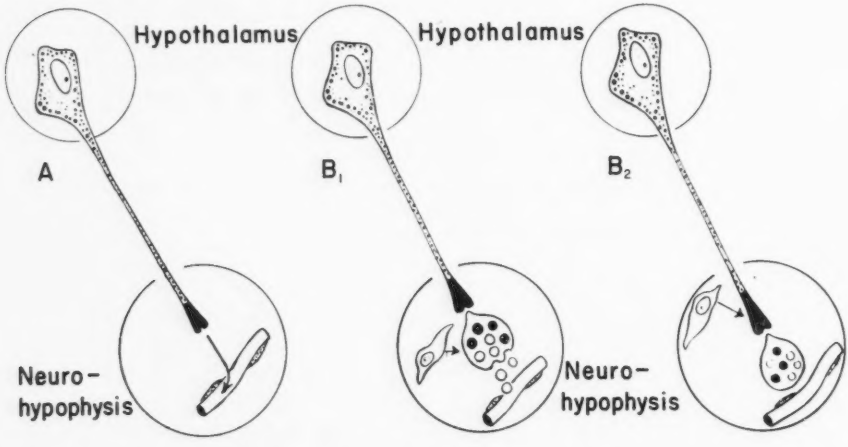


FIG. 5. Diagrammatic representation of the concepts of synthesis and release of posterior lobe hormones. (A) Advanced by Leveque and Scharrer as an explanation of the role of hypothalamic neurons in hormone synthesis. (B_1) and (B_2) Two possible explanations for the increased mitotic activity in dehydration. (B_1) Introduction of a factor from the pituicyte in the separation of hormones from a "Carrier substance". Large tear-shape structure between axon and blood vessel represents "Carrier substance". Small solid circles represent oxytocin and small open circles represent vasopressive-antidiuretic factor. Arrow shows site of activity of postulated pituicyte factor (B_2) same as (B_1) except that arrow indicates that pituicyte factor may cause release from axon.

REFERENCES

1. LEVEQUE, T. F. AND E. SCHARRER: *Endocrinology* **52:** 436. 1953.
2. SCHARRER, E. AND B. SCHARRER: Handb. Mikr. Anat. des Menschen, 6/5:953 Springer-Verlag, Berlin, 1954.
3. GERSH, I.: *Am. J. Anat.* **64:** 407. 1939.
4. GERSH, I. AND C. MC. BROOKS: *Endocrinology* **28:** 6. 1941.
5. SELYE, H. AND C. E. HALL: *Anat. Rec.* **86:** 579. 1943.
6. STUTINSKY, F.: *C. R. Soc. Biol.* **145:** 367. 1951.
7. STUTINSKY, F.: *C. R. Soc. Anat.* Geneva, April, 1954.
8. RENNELS, E. G. AND G. A. DRAGER: *Anat. Rec.* **122:** 193. 1955.
9. ORTMANN, R.: *Z. Zellforsch.* **36:** 92. 1951.
10. BILLENSTIEN, D. AND T. F. LEVEQUE: *Endocrinology* **56:** 704. 1955.
11. DICKER, S. E. AND J. NUNN: *J. Physiol.* **136:** 235. 1957.

STUDIES ON SOME HORMONAL AND GENETIC FACTORS REGULATING NITROGEN RELEASE FROM THE ISOLATED MOUSE DIAPHRAGM[1]

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ABSTRACT

Nitrogen release by the isolated diaphragm was greater for tissues taken from I/Fn than for those taken from A/Fn strain mice. Previous treatment of the mice with thyroxine enhanced the rate of *in vitro* release of the A/Fn tissues but had no significant effect on the I/Fn. It is suggested that I/Fn mice normally release more theyroid hormone than the A/Fn and that this accounts in part for the different rates of protein catabolism observed earlier.

THE isolated diaphragm lends itself nicely to the study of nitrogen release from tissues and the factors which control it. More than most tissues, it may be prepared for *in vitro* experiments with minimal damage to its component cells. There is now substantial evidence that nitrogenous compounds are released from isolated tissues as part of normal metabolic processes under the influence of the endocrine system (1, 2, 3).

Earlier studies from this laboratory have revealed large metabolic differences among several strains of mice. We have postulated that the genetic composition of our strains of mice determines the pattern of endocrine output or tissue sensitivity and that this in turn determines the metabolic patterns observed with each strain. The genetic determination of endocrine patterns has been demonstrated in many laboratories, and there is no question today about the endocrine control of metabolism. Our problem has been to delineate the exact nature of these endocrine patterns in our mice since direct measurements of hormone output are somewhat difficult in such a small animal.

Nitrogen balance experiments with intact animals have shown (4) that the I/Fn strain mouse ordinarily turns over and excretes nitrogen more rapidly than the A/Fn strain animal when fed ad libitum. It seemed appropriate to investigate this problem at the tissue level employing the isolated diaphragm. This appeared particularly promising because of the demonstration by Kline (2) that the nitrogen release from the diaphragm can be influenced by suitable manipulation of the endocrine system. Be-

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cause of its thinness the mouse diaphragm seemed particularly suitable for *in vitro* studies, and few changes were required to adapt Kline's techniques to the mouse.

MATERIALS AND METHODS

The study was carried out on tissues removed from 6-month-old male mice of the A/Fn and I/Fn strains. The animals had access to food and water until removal of the diaphragm under Nembutal anesthesia. The tissues were kept moist and cold while connective tissue was trimmed as far as possible. The hemidiaphragms were weighed on a torsion balance and then incubated in 4 ml. of Krebs-Ringer bicarbonate solution containing 0.2% glucose. The vessels were gassed for 2 minutes with 95% oxygen and 5% CO_2, stoppered, placed in a water bath at 38° C. and shaken at a rate of 120 cycles /minute.

After a 15 minute equilibration period, as suggested by Kline (2), an aliquot of medium was removed by means of a Lang-Levy constriction pipette. Incubation was then continued for an additional three hours. After centrifugation, the nitrogen content of the medium at 15 minutes and at 3 hours and 15 minutes was determined by the method of Folin and Ciocalteu (5), and was expressed on the basis of wet weight of tissue. Simultaneous application of the Folin-Ciocalteu method and the micro Kjeldahl procedure to a substantial quantity of the medium after $3\frac{1}{4}$ hours of incubation with tissue gave a conversion factor for transforming tyrosine values into total nitrogen values. To minimize the effects of subtle variations in technique, animals from groups to be compared were run at the same time.

Crystalline L-thyroxine was administered subcutaneously to the experimental groups at a dose of 10 mg./kg. body weight each day for seven days before removal of the diaphragm. Other hormones were used as described in the text.

RESULTS

Nitrogen released by diaphragms of untreated I strain mice was almost 30% greater than the release from untreated A strain diaphragms (Table 1). Administration of thyroxine daily for seven days caused a large, significant increase in nitrogen release of A strain mice and a small, statistically insignificant increase in the I strain animals. The difference in nitrogen release from tissues of thyroxine-treated animals of the two strains was less than half the difference between their uninjected controlled groups and was no longer statistically significant. A single dose of thyroxine (4 mg./kg. body weight) given two days before sacrificing the animals was without effect on the rate of nitrogen release.

TABLE 1. EFFECT OF REPEATED THYROXINE INJECTIONS ON RATE OF NITROGEN RELEASE FROM ISOLATED DIAPHRAGMS OF TWO STRAINS OF MICE

Strain	n	Control μg./100 mg.†	n	Injected μg./100 mg.	Difference	p
A	15	395 ± 17*	12	523 ± 18	128 ± 25	<0.001
I	15	512 ± 24	12	571 ± 17	59 ± 30	>0.05
Difference		117 ± 30		48 ± 25		
P		<0.001		>0.05		

† μg. nitrogen/100 mg. wet tissue/3 hrs.
* Standard error.

Insulin (1 unit/kg. body weight) given intraperitoneally one hour before sacrifice and adrenal cortical extract (20 ml./kg. body weight) given three hours before sacrifice had no effect on the rate of nitrogen release from the diaphragms of the two strains of mice. Growth hormone[3] administered one hour before sacrifice gave a variable response. A dose of 10 mg./kg. body weight frequently induced an increase in the rate of nitrogen release. On the other hand, a dose of 50 mg./kg. body weight occasionally caused a decrease in nitrogen release. The effects, however, were by no means consistent.

DISCUSSION

There is no certainty at present as to the physiological significance of the protein released by the incubated diaphragm. If one assumes that this protein outflow is characteristic of all muscle, the total amount released per 24 hours far exceeds the amount of nitrogen normally excreted by the intact animal. Obviously only a small portion, at most, of the released nitrogen undergoes destruction and excretion. Furthermore the amount of protein released by muscle in one day, if the diaphragm is representative of other muscle, exceeds the total amount of protein normally present in the plasma of the mouse.

If the rate of protein release from the isolated diaphragm is any indication of the magnitude of protein turnover in the whole animal, then the data on untreated A and I strain mice agree with earlier observations by Fenton and Marsh (4) that under the usual conditions of studying nitrogen balance the I strain mouse excreted more nitrogen per day than did the A strain. The observations in the present paper indicate that the protein release from I strain diaphragm is significantly greater than that from A strain tissue.

The thyroxine effect demonstrated here extends the original observation by Kline (2) that diaphragms taken from thyroidectomized rats release protein at a reduced rate and that thyroxine treatment of such animals restores the rate of release to normal. A very large dose of thyroxine was chosen in the hope of pushing the N-releasing system of both strains to its limit. The data show that this limit is virtually the same in the two strains. In this light the significant difference in N-release by tissues of uninjected animals might indicate that the I strain mouse normally operates with a large endogenous thyroxine output which makes the exogenous dose less effective than it is in the A strain. Alternatively, it may be that the I strain mouse is relatively resistant to the metabolic effects of exogenous thyroxine. This would leave unexplained the strain difference in N release shown by the diaphragms of uninjected animals.

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[3] The growth hormone used in this work was a gift from the Endocrinology Study Section, National Institutes of Health.

REFERENCES

1. Bernheim, F. and M. L. C. Bernheim: *J. Biol. Chem.* **163:** 203. 1946.
2. Kline, D. L.: *Endocrinology* **45:** 596. 1949.
3. Kaufmann, E. and E. Wertheimer: *Am. J. Physiol.* **190:** 133. 1957.
4. Fenton, P. F. and J. M. Marsh: *J. Nutrition* **60:** 465. 1956.
5. Folin, O. and V. Ciocalteu: *J. Biol. Chem.* **73:** 627. 1927.

STUDIES ON THE MECHANISM OF HYPOTHALAMIC CONTROL OF THYROTROPIN SECRETION: COMPARISON OF THE SENSITIVITY OF THE HYPOTHALAMUS AND OF THE PITUITARY TO LOCAL CHANGES OF THYROID HORMONE CONCENTRATION[1]

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ABSTRACT

The microinjection of graded doses of systemically ineffective amounts of thyroxine into the anterior hypothalamus or pituitary of the rat was utilized to study the mechanism of the hypothalamic control of thyrotropin secretion. The rate of secretion of endogenously labeled hormone from the thyroid gland was considered to reflect thyrotropin secretion.

Thyroxine introduced into either the anterior hypothalamic "thyrotropin area" or into the anterior pituitary would inhibit thyrotropin secretion. The amount of thyroxine required to decrease TSH secretion was approximately equivalent for both areas. Less than 1% of labeled thyroxine introduced into the anterior hypothalamus reached the anterior pituitary, making it extremely unlikely that thyroxine injected into the hypothalamus is first transported to the adenohypophysis where its inhibitory effect is exerted.

It thus appears that two separate receptor sites, one in the anterior hypothalamus and one in the adenohypophysis, are sensitive to local concentrations of thyroid hormone and act as the thermostats in the servo mechanism which maintains a stable level of circulating thyroid hormone.

IN TWO previous papers from this laboratory (1, 2), it has been shown that systemically ineffective amounts of thyroxine can inhibit thyrotropin secretion when injected directly into either the anterior hypothalamus or pituitary. It was postulated that there might be two separate receptor sites, one in the anterior hypothalamus and one in the anterior pituitary which are sensitive to local alterations of thyroxine concentration. These would act as the "thermostats" in the servo mechanism which maintains a constant level of circulating thyroid hormone.

In order to establish this hypothesis, it was necessary to exclude the possible diffusion of thyroxine injected into the hypothalamus to the anterior pituitary where it would exert its inhibitory effect. In the present investigation the diffusion of labeled thyroxine following intrahypothalamic injec-

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[1] This is an abridgement of the 1959 Van Meter Prize Award paper of the American Goiter Association.

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tion was studied. A comparison of the sensitivity of the hypothalamic and hypophyseal centers to graded doses of locally introduced thyroxine was also made.

MATERIALS AND METHODS

One hundred six adult male Holtzman rats, weighing 220 to 326 gm., were employed. Animals were fed a Remington low iodine diet for at least 7 days in order to obtain a high uptake of radioiodine by the thyroid. A number 27 hypodermic needle was inserted into the anterior hypothalamus or the pituitary with a stereotaxic instrument and fixed to the skull by 4 screws as previously described (1). At autopsy the exact position of the needle tip was studied with a dissecting microscope and with serial histological sections. Four to five days after insertion of the needle, 20 μC of radioiodine were injected intraperitoneally. Beginning 24 hours later, 30 mg. of propylthiouracil were injected subcutaneously daily at rotated sites in order to block the reaccumulation in the thyroid of I^{131} liberated from degradation of endogenously labeled thyroxine. Determination of radioactivity in the thyroid region was made 5 times daily by means of an externally placed scintillation counter under light ether anesthesia and each count was plotted as % of the initial 24-hour uptake on semilog paper. After obtaining a straight line containing more than 5 individual points, 0.02 ml. saline or graded doses of thyroxine (0.05, 0.10, 0.15, 0.20 and 0.25 μg. L-thyroxine) contained in 0.02 ml. saline were injected into the anterior hypothalamus or pituitary through the needle previously fixed to the skull. The dead space of each needle was taken into account when calculating the volume injected. As previously described (1), a significant decrease in the slope of the plotted thyroidal radioactivity following thyroxine injection was interpreted as indicating depressed thyroidal secretion.

0.25 μg. labeled thyroxine (0.2 μC) were injected into the anterior hypothalamus in animals in which the fate and distribution of radioactive thyroxine were studied. Thirty minutes and 4 hours after the injection, the animals were autopsied and the radioactivity of the hypothalamus, pituitary and the remainder of the brain was separately measured.

RESULTS

I. *Comparison of Intrahypothalamic and Intrapituitary Injection of Graded Doses of Thyroxine on Thyroid Hormone Secretion*

The results are summarized in Table 1 and Figure 1. All animals which received saline alone exhibited no change in the exponential rate of thyroidal secretion observed at the beginning of the experiment. It can be seen that the sensitivity of both the hypothalamic and pituitary receptor areas were approximately equal, although there might have been a slightly greater sensitivity of the pituitary.

The delayed effect of intrahypothalamic thyroxine injection on thyroid hormone release, which was conspicuous in previous experiments, was also noted in most of the animals in this study. The duration of inhibition following thyroxine was approximately equal in both the hypothalamic and pituitary groups (Table 1).

The needle tips were in the anterior hypothalamic thyrotropin area of the hypothalamus or in the anterior pituitary in all animals (Fig. 2) as confirmed at autopsy. The animals which received 0.25 μg. of thyroxine in

TABLE 1. TABULATION OF THE EXPERIMENT IN WHICH THE EFFECT ON THYROIDAL SECRETION OF SALINE AND GRADED DOSES OF T4 INJECTED INTO THE ANTERIOR HYPOTHALAMUS OR PITUITARY WAS STUDIED. ABOUT THE SAME EFFECT WAS PRODUCED BY INJECTION OF THYROXINE INTO EITHER PLACE

	No. of animals	Exhibiting depressed thyroid secretion		Duration of inhibition (hrs.)
		No. of animals	Per cent	
Hypothalamus group				
1. Needle only	3	0	0	0
2. Saline	4	0	0	0
3. 0.05 μg. T4	8	1	13	6
4. 0.10 μg. T4	5	2	40	18.5±8.0
5. 0.15 μg. T4	5	2	40	14.5±5.0
6. 0.20 μg. T4	5	3	60	18.0±0.7
7. 0.25 μg. T4	18	16	89	20.6±1.1
Pituitary group				
1. Needle only	2	0	0	0
2. Saline	4	0	0	0
3. 0.05 μg. T4	7	1	14	24.5
4. 0.10 μg. T4	4	3	75	18.6±6.3
5. 0.15 μg. T4	5	3	60	6.0±0.7
6. 0.20 μg. T4	7	7	100	22.1±3.7
7. 0.25 μg. T4	4	4	100	20.6±6.0

both groups have been reported in the first paper in this series (1). Thus the 2 animals receiving this dose in the hypothalamic group in which no response was produced had the tips of the needles penetrating slightly

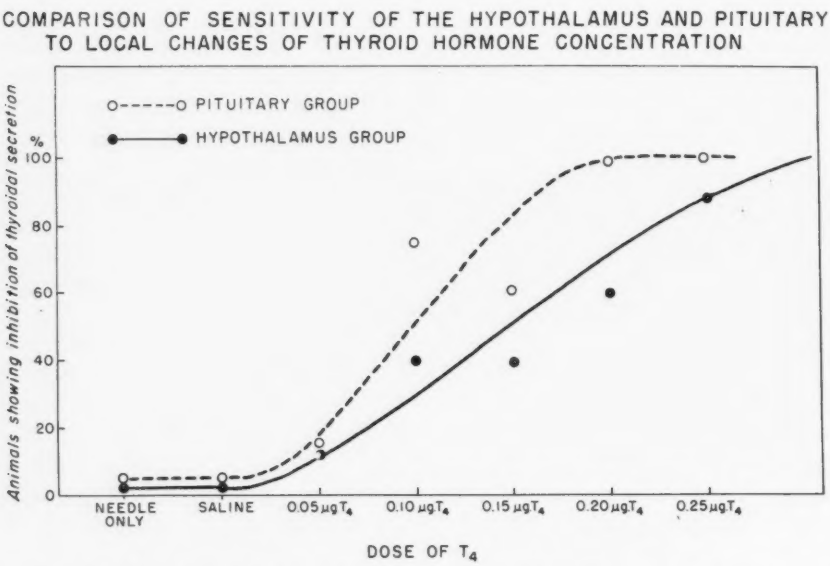

FIG. 1. Effect of graded doses of thyroxine injected into the anterior hypothalamus or the pituitary on thyroid secretion rate. Both groups demonstrate an increasing percentage of inhibition with increasing doses of thyroxine.

through the basal border of the hypothalamus and thyroxine may have escaped into the subarachnoid space rather than being injected into the hypothalamus itself.

II. *The Fate and Distribution of Radioactive Thyroxine Injected into the Anterior Hypothalamus.*

As shown in Figure 3, 30 minutes after intrahypothalamic injection of labeled thyroxine, about 30% of the radioactivity remained in the hypothalamus while 30–60% of the injected thyroxine had diffused to the remainder of the brain. On the other hand, only 0.3 to 0.9% of the radioactivity was present in the pituitary at that time. Four hours after thyroxine injection the radioactivity had declined markedly in all areas, averaging about 4, 0.05 and 7% of the administered dose in the hypothalamus, pituitary, and remainder of the brain respectively. Thus the maximal amount of thyroxine reaching the pituitary after intrahypothalamic injection would be only 1% of the administered dose.

DISCUSSION

Most investigators have found that lesions in the "thyrotropin area" of the anterior hypothalamus (3, 4), centrally located between the paraventric-

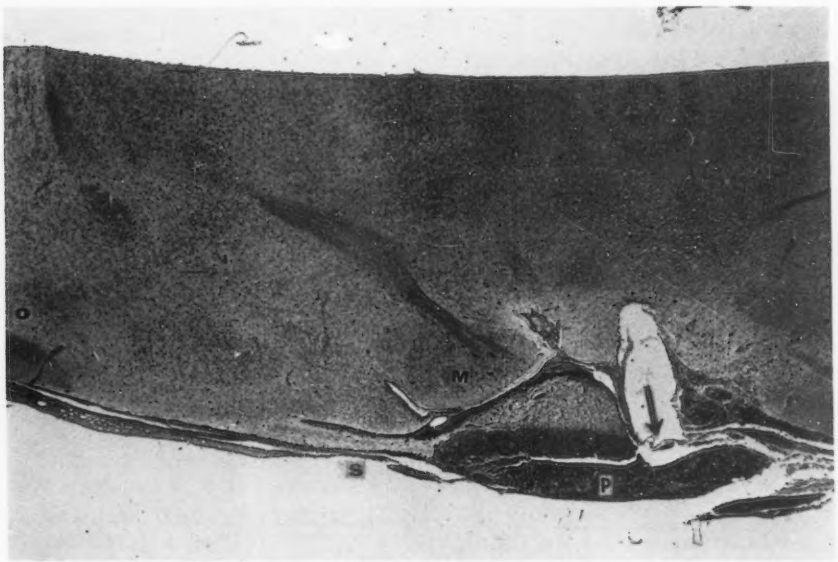

FIG. 2. Photograph of a sagittal section of the hypothalamus and pituitary of a thyroxine injected animal. The tip of the needle was in the anterior pituitary. Arrow indicates needle tip. P = Pituitary; O = Optic Nerve; S = Pituitary Stalk; M = Mammillary Body.

ular nucleus and the median eminence, consistently result in a diminution of pituitary thyrotropin secretion. Lesions in other areas of the hypothalamus (3), removal of the neocortex (5), or destruction of the amygdala (6) do not significantly depress thyrotropin secretion. These results suggest that the anterior hypothalamus may be a primary center controlling thyrotropin secretion, although the participation of other parts of the central nervous system has not yet been completely excluded.

In the two previous papers from this laboratory (1, 2), it was postulated that two separate areas, one in the anterior hypothalamus and one in the anterior pituitary, might be sensitive to local changes in thyroxine concentration and thus alter thyrotropin secretion by the pituitary. In this study it has been shown that both these areas are approximately equally sensitive to local changes in thyroxine concentration. It has also been shown that less than 1% of the thyroxine injected into the anterior hypothalamus

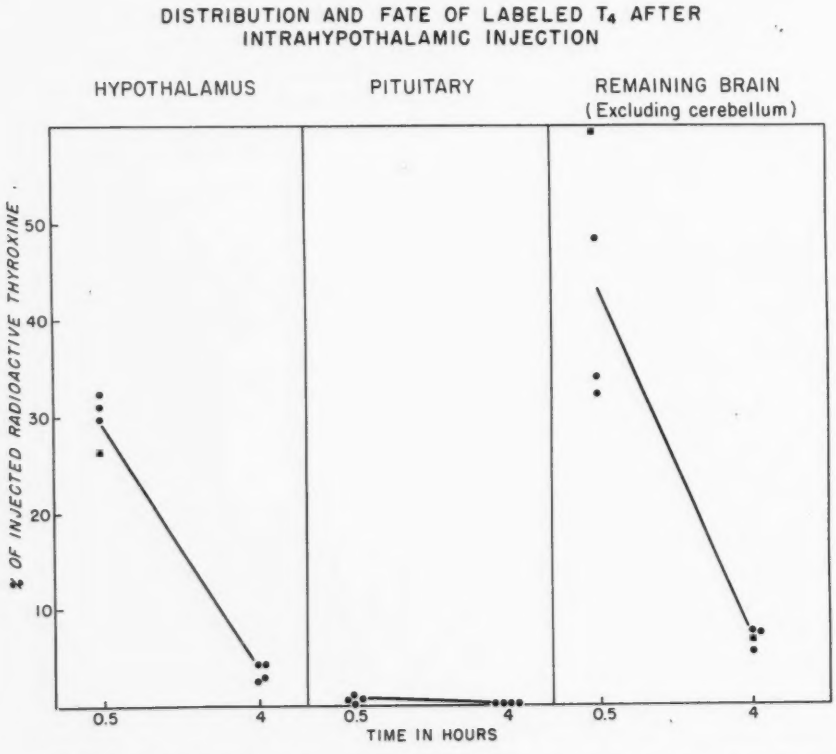

FIG. 3. Fate of radioactive thyroxine injected into the anterior hypothalamus. Radioactivity in the hypothalamus, pituitary and the remainder of the brain were separately measured 0.5 and 4 hours after the injection of 0.25 μg. labeled thyroxine with an activity of 0.2 μC.

diffuses to the anterior pituitary. It thus seems extremely unlikely that the inhibition of thyrotropin secretion produced by the injection of thyroxine into the anterior hypothalamus can be explained by its being carried to the anterior pituitary, there to exert its inhibitory effect.

The minimum effective dose of thyroxine which inhibited thyrotropin secretion when introduced directly into the pituitary was 0.05 μg. The amount that could be calculated to be present in the pituitary from the injection of a systemically effective dose of 5 μg. of thyroxine would be only 0.0003 μg. Approximately the same difference in concentration between local and systemic injections of thyroxine would also be the case for the anterior hypothalamus.

Two possible explanations might be considered for this 100-fold difference in calculated sensitivities. 1) Thyroxine injected systemically would be distributed equally throughout the "receptor" area via the blood vessels. Thyroxine injected directly into the pituitary or hypothalamus might not distribute equally throughout the effective sites and therefore larger amounts would be required to inhibit thyrotropin secretion than would be required via the systemic route. 2) Thyroxine injected directly might diffuse along the inserted needle and thus the actual concentration at the receptor site would be much smaller than calculated from the amount injected. This might be particularly true when the materials were injected into the hypothalamus since the injection is made in the midline and the solution could easily escape into the third ventricle and be carried away in the cerebro-spinal fluid. One other consideration is that there is a concentration of thyroxine and triiodothyronine in both the hypothalamus and pituitary of rabbit and guinea pig, as has been demonstrated by Ford and Gross (7, 8). Although no significant concentration of thyroxine has yet been found in rat pituitary or hypothalamus (9), triiodothyronine is concentrated in both areas (10). It is therefore possible that the actual concentration in the receptor area of the hypothalamus and pituitary might be much greater than would be expected by assuming equal distribution throughout all tissues.

REFERENCES

1. Yamada, T. and M. A. Greer: *Endocrinology* **64**: 559. 1959.
2. Yamada, T.: *Endocrinology* **65**: 216. 1959.
3. Greer, M. A. and H. Erwin: *J. Clin. Invest.* **33**: 938. 1954.
4. Ganong, W. F., D. S. Fredrickson and D. M. Hume: *Endocrinology* **57**: 355. 1955.
5. Greer, M. A. and H. F. Shull: *Proc. Soc. Exper. Biol. & Med.* **94**: 565. 1957.
6. Yamada, T. and M. A. Greer: Unpublished Data.
7. Ford, D. H. and J. Gross: *Endocrinology* **63**: 549. 1958.
8. Ford, D. H. and J. Gross: *Endocrinology* **62**: 416. 1958.
9. Taurog, A., G. W. Harris, W. Tong and I. L. Chaikoff: *Endocrinology* **59**: 34. 1956.
10. Ford, D. H., S. Kantounis and R. Lawrence: *Endocrinology* **64**: 977. 1959.

GROWTH IN THE HYPOPHYSECTOMIZED RAT SUSTAINED BY PITUITARY GRAFTS

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ABSTRACT

Grafting of 4 pituitaries under the kidney capsule of the weanling, hypophysectomized rat sustained suboptimal somatic growth at about two-thirds the normal rate. Grafted-hypophysectomized rats have thyroids and gonads which are larger than those of hypophysectomized controls, except for 10 percent of the males which, in addition, have varying degrees of sustained testicular function and size. It is concluded that in the rat, somatotropin production and release are not completely dependent upon an intact hypothalamic-pituitary system.

THERE are available extensive observations concerning the hypothalamic dependence of the anterior pituitary for optimal adrenotropic, thyrotropic, and gonadotropic function (1). We have, however, little detailed information regarding the relationship of the hypothalamus to the somatotropic function of the pituitary. Greep (2) observed continued growth in young rats receiving intrasellar pituitary grafts. However, since these grafts also exhibited normal gonadotropic function, it may be inferred that their relationship to the adjacent hypothalamus had become reestablished. Martinovitch and Vidovic (3) referred in passing to continued growth in the pituitary-grafted hypophysectomized rat but provide no quantitative data. May (4) presented autopsy data on 4 hypophysectomized rats bearing pituitary grafts which indicate some sustained growth. Goldberg and Knobil (5) reported "slightly greater growth" in grafted-hypophysectomized rats than in hypophysectomized controls and state that whether this is "related to secretion of growth hormone is not clear."

This report presents detailed studies of the growth pattern following hypophysectomy in the young rat bearing multiple pituitary grafts. The data demonstrate that substantial somatic growth can be supported by such grafts despite their failure to maintain optimal thyroid, adrenal, and gonadal function.

MATERIALS AND METHODS

Weanling Sprague-Dawley male or female rats were hypophysectomized by the parapharyngeal approach at 22 days of age. One to 3 days later, 4 whole pituitary glands from donors of the same age and sex as the recipient were placed underneath the left

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kidney capsule. A total of 150 hypophysectomized, 135 hypophysectomized and grafted, and 100 intact normal control rats, comprising 13 separate experimental series have been studied. Detailed growth records of the hypophysectomized-grafted rats were kept and compared in each series with those of hypophysectomized and intact controls.

Autopsies were performed at varying intervals on equal numbers of intact, grafted-hypophysectomized, and non-grafted-hypophysectomized rats which were received at the same time and maintained in adjacent cages in the laboratory. Tissues were fixed in Bouin's solution and stained by hematoxylin and eosin. The pituitary grafts were recovered at autopsy and their viability assessed by cytological study. The histological status of the thyroids, adrenals, and gonads was carefully appraised. The completeness of the hypophysectomy was determined by gross examination under good illumination and 10-fold magnification. Residual sellar contents were serially sectioned in any doubtful cases. Any incompletely hypophysectomized animals were eliminated. Skeletal x-rays were taken of representative series to determine the comparative degree of osseous development of experimentals and controls.

RESULTS AND DISCUSSION

Within one week following the operative procedures, the hypophysectomized rat was readily distinguishable from the grafted-hypophysectomized animal. The characteristic flaccidity and furriness of the hypophysectomized animal were not seen in the grafted animal. The latter had excellent muscle tone and the hair-coat was coarse and thick.

Quantitative data from 4 of the larger experimental series are presented in Table 1. Figure 1 presents the highly characteristic growth curves from a selected series of female rats. Figure 2 presents radiological evidence that the greater body weight of the grafted animals is accompanied by true skeletal development and increased body length. It is clear from these data that somatic growth is sustained in the grafted animals, but this proceeds at approximately two-thirds of the growth rate seen in intact, control animals.

It is especially significant that the pituitary grafts can support substantial somatic growth without a comparable degree of trophic action on the thyroids and gonads (Table 2). That such graft-supported growth is not optimal may, in fact, be attributable to the associated thyroid impairment.

The grafted-hypophysectomized female rat invariably exhibits a marked atrophy of the ovary and uterus, although this is neither grossly nor histologically as profound as that seen in the hypophysectomized control (Table 2). In contrast, we have noted that 10% of the grafted-hypophysectomized male rats show varying degrees of testicular development which, in some cases, is grossly and histologically indistinguishable from that seen in the intact male rat. That this is not attributable to incomplete hypophysectomy is manifest from the fact that the thyroids and adrenals of such animals show atrophy comparable to that seen in other hypophysectomized-grafted rats. These observations in the male are not

TABLE 1. BODY WEIGHT DATA IN HYPOPHYSECTOMIZED, HYPOPHYSECTOMIZED-GRAFTED, AND INTACT RATS*

Series	Endocrine status	Age in weeks										
		4	5	6	7	8	9	10	11	12	14	17
A ♀ ♀	Hypophysectomized	55 ± 3	63 ± 5	68 ± 4	70 ± 6	75 ± 4	80 ± 7	92 ± 7	96 ± 8	99 ± 5	—	—
	Hypophysectomized and Grafts	58 ± 4	80 ± 7	107 ± 7	120 ± 10	126 ± 11	135 ± 10	142 ± 8	154 ± 12	161 ± 13	—	—
	Intact	60 ± 3	100 ± 7	135 ± 9	158 ± 11	172 ± 9	196 ± 5	204 ± 6	215 ± 12	222 ± 10	—	—
B ♀ ♀	Hypophysectomized	57 ± 4	62 ± 6	70 ± 5	72 ± 4	78 ± 7	81 ± 4	90 ± 4	100 ± 9	100 ± 4	—	—
	Hypophysectomized and Grafts	58 ± 3	184 ± 6	105 ± 8	117 ± 11	125 ± 9	136 ± 11	146 ± 7	156 ± 5	160 ± 8	—	—
	Intact	61 ± 5	105 ± 9	141 ± 6	155 ± 8	180 ± 5	192 ± 8	206 ± 9	212 ± 10	220 ± 12	—	—
C ♀ ♀	Hypophysectomized	52 ± 5	60 ± 5	69 ± 4	70 ± 6	—	—	—	95 ± 9	—	102 ± 9	115 ± 7
	Hypophysectomized and Grafts	55 ± 6	71 ± 4	94 ± 5	111 ± 9	—	—	—	171 ± 8	—	197 ± 9	221 ± 15
	Intact	60 ± 5	98 ± 7	140 ± 9	170 ± 18	—	—	—	282 ± 20	—	334 ± 30	358 ± 26
D ♂ ♂	Hypophysectomized	54 ± 4	61 ± 4	68 ± 6	—	96 ± 7	—	—	97 ± 12	—	107 ± 15	—
	Hypophysectomized and Grafts	55 ± 8	70 ± 5	107 ± 6	—	143 ± 8	—	—	176 ± 11	—	198 ± 12	—
	Intact	61 ± 6	92 ± 5	141 ± 9	—	183 ± 11	—	—	217 ± 15	—	232 ± 12	—

* Each series comprises 12 to 15 animals per experimental group.
Figures represent means and standard deviations of body weights in grams as determined at close of period indicated.

detailed since they are quite comparable to those described by Goldberg and Knobil (5).

It is noteworthy that although the weights of the thyroid and ovaries in the hypophysectomized-grafted animals indicate a major degree of hypophyseal deficiency, these organs are larger than corresponding organs of the hypophysectomized controls. One is led to infer that the ectopic pitui-

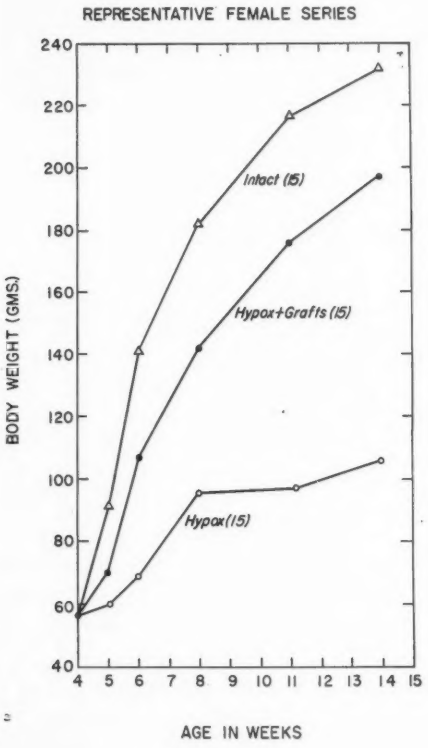

FIG. 1. Growth curves of hypophysectomized, hypophysectomized-grafted and intact female rats. (Numbers in parenthesis indicate number of animals per group.)

tary may continue to exert a sub-optimal trophic action upon these target organs. Functional tests reported for rats and mice by others have also indicated considerable but sub-optimal thyrotropic as well as adrenotropic function in ectopic pituitary tissue (6, 7, 8).

Morphological study of each of the 4 recovered pituitary grafts indicates quite clearly that the degree of functional pituitary impairment observed in these animals is not attributable to a simple quantitative deficiency of viable and well vascularized anterior lobe tissue.[1]

Pituitary stalk-section has been reported to produce palliative regression

[1] Detailed morphological and cytological analysis of these grafts is to be reported upon elsewhere by Dr. Oscar Vilar.

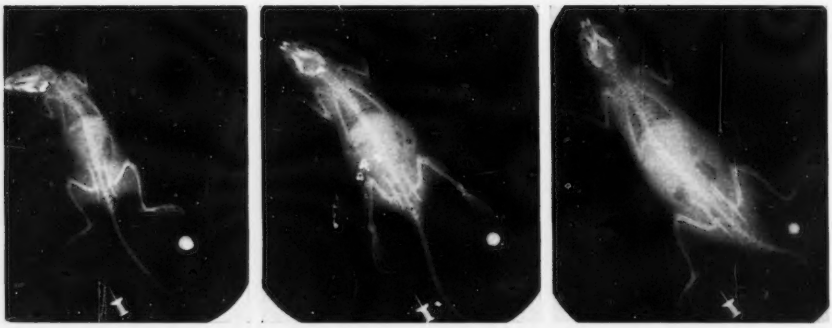

FIG. 2. Comparative x-rays of hypophysectomized, hypophysectomized-grafted, and intact rats, arranged left to right; all animals are 100 days old.

TABLE 2. QUANTITATIVE DATA IN A REPRESENTATIVE SERIES OF GRAFTED-HYPOPHYSECTOMIZED RATS*

Status	Initial body weight (gm.)	Final body weight (gm.)	Adrenals (mg.)	Thyroid (mg.)	Ovaries (mg.)	Uterus (mg.)
Hypophysectomized	56 ± 4	113 ± 5	12 ± 3	5 ± 1	3 ± 1	16 ± 4
Hypophysectomized and grafted	57 ± 4	196 ± 8	13 ± 2	9 ± 1	10 ± 2	31 ± 5
Normal controls	63 ± 5	227 ± 10	60 ± 4	13 ± 2	68 ± 8	240 ± 20

* 10 rats per group; 100 days old at autopsy; values represent mean and standard deviations.

of advanced breast cancer in women (9). These effects are stated to be qualitatively comparable to those obtained by hypophysectomy. However, Mühlbock and Boot (10) have demonstrated that subcutaneous pituitary grafts will markedly increase the incidence of breast cancer in a strain of mice which lacks the mammary tumor agent and, therefore, exhibits a very low spontaneous incidence of such tumors. These authors interpret their findings in the light of the known production of prolactin by the ectopic pituitary (11). However, the present studies indicate that such pituitary grafts could bring about their tumor-enhancing effect by also producing growth hormone.

Acknowledgment

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REFERENCES

1. HARRIS, G. W.: The Neural Control of the Pituitary Gland, Williams and Wilkins, Baltimore, 1955.
2. GREEP, R. O.: *Proc. Soc. Exper. Biol. & Med.* **34**: 754. 1936.

3. MARTINOVITCH, P. M. AND V. L. VIDOVIC: *Bull. Inst. Nuclear Sciences*, Boris Kidrick; No. 48, 131. Aug. 1953.
4. MAY, R. M.: *C. R. Soc. Exper. Biol.* **124:** 920. 1937.
5. GOLDBERG, R. C. AND E. KNOBIL: *Endocrinology* **61:** 742. 1957.
6. FORTIER, C. AND H. SELYE: *Am. J. Physiol.* **159:** 433. 1949.
7. MCDERMOTT, W. V., E. G. FRY, J. R. BROBECK AND C. N. H. LONG: *Yale J. Biol. & Med.* **23:** 52. 1950.
8. GREER, M. A.: *J. Clin. Endocrinol. and Metab.* **12:** 1259. 1952.
9. BUXTON, P. H., F. L. DAVIES, A. M. JELLIFFE, K. M. JONES, V. LOGUE, J. D. N. NABARRO AND G. WALKER: *in Endocrine Aspects of Breast Cancer*, p. 58. Williams and Wilkins, Baltimore, 1958.
10. MÜHLBOCK, O. AND L. M. BOOT: *Cancer Research* **19:** 402. 1959.
11. EVERETT, J. W.: *Endocrinology* **54:** 685. 1954.

VISUALIZATION OF STEROID-3β-OL-DEHYDROGENASE ACTIVITY IN TISSUES OF INTACT AND HYPOPHYSECTOMIZED RATS[1]

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ABSTRACT

Dehydrogenation of dehydroepiandrosterone, a 3β-hydroxysteroid, is demonstrable in all types of steroid-producing cells in the rat adrenal gland, ovary and testis, as well as in some of the oviduct epithelium, when sections are incubated in a phosphate buffer (pH 7.1–7.4) solution containing substrate, propylene glycol, DPN, nicotinamide and the tetrazolium salt, Nitro-BT. No activity toward this substrate is evident in liver or kidney. After cold acetone treatment, activity is severely inhibited in the follicular theca interna but not in the other sites. Steroid-3β-ol dehydrogenase appears more labile than the widely-distributed DPN-dependent dehydrogenase, lactic dehydrogenase.

Enzymatic activity remains conspicuous in rat ovary and adrenal cortex after hypophysectomy, declining after 1–2 months only in those residual steroid-producing cells that have become severely atrophied (ovarian interstitial cells, inner zones of the adrenal cortex). Treatment with ACTH restores adrenocortical activity in long-term hypophysectomized rats.

Some dehydrogenation of 17-hydroxysteroids occurs in the same tissues and also in liver and kidney.

BIOCHEMICAL methods have been used to demonstrate and characterize a diphosphopyridine (DPN)-dependent dehydrogenase capable of oxidizing Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids in adrenal cortex, corpus luteum, testis and placenta (1, 2, 3). Recently Wattenberg (4) adapted the Farber *et al.* (5) histochemical technique for visualizing DPN-dependent dehydrogenases to the demonstration of steroid-3β-ol dehydrogenase. An unfixed tissue section is incubated in a medium containing the substrate, e.g. dehydroepiandrosterone (DHA), plus DPN, a tetrazolium salt and buffer. Hydrogen ions removed from the substrate by the enzyme are transferred to DPN, then from DPNH to the tetrazole by a portion of the electron transport system related to DPNH-cytochrome-*c* reductase (6). Therefore the method does not demonstrate the activity of the specific

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[2] Recipient of a Portuguese government traveling fellowship through the Instituto da Alta Cultura and of a grant from the Foundation Calouste Goulbenkian, 1958–1959. Permanent address: Instituto de Anatomia Patologica, Faculdade de Medicine, Lisbon, Portugal.

TABLE 1. DATA ON HYPOPHYSECTOMIZED FEMALE RATS AND THEIR CONTROLS*

Treatment	No. rats	Days after operation	Body weight, gm.	Organ weight, mg./100 gm. body weight	
				Paired adrenals	Paired ovaries +oviducts
None	4	—	254	25.8	42.3
Hypox	1	3	215	21.8	49.0
	2	15	218	13.5	33.5
	2	28	211	9.7	25.6
	2	50	191	7.9	22.0
Hypox. +5 U. ACTH/d.	1	70+ 1½	216	7.6	27.3
	1	70+ 3½	198	9.6	14.4
	1	70+ 8	212	13.7	22.2

21.3

* Purchased from the Charles River Breeding Labs., Brookline, Mass.

dehydrogenase directly but that of a sequence of enzymes. Presumably the sequence is identical for all DPN-dependent enzyme systems subsequent to the specific dehydrogenase. If the enzyme or the formazan (reduced tetrazole) produced is not soluble, however, accurate cellular (but not intracellular) localization of the specific enzyme appears possible (4, 7).

We have developed a slightly different medium that appears to demonstrate activity readily and in some locations not described in the cited studies (1, 4). We have made a preliminary survey of the distribution of the enzyme system in the steroid-producing glands of several laboratory rodents, comparing it with that of a generally-distributed DPN-dependent enzyme, lactic dehydrogenase (8). The present report summarizes a study made of organs of normal adult male and female rats and of hypophysectomized female rats.

METHODS

The adrenal glands, and testes or ovaries and adnexa of 18 normal adult rats of both sexes were examined. In a few cases, pieces of liver and kidney were also studied. In addition, the adrenals and ovaries of 10 hypophysectomized female rats were compared with those of 4 normal controls (Table 1). Three of the animals hypophysectomized 10 weeks ealier received daily subcutaneous injections of 5 U. ACTH gel[3] and were killed after 2, 4 and 8 injections. The completeness of hypophysectomy was confirmed at autopsy by examination of the sella turcica.

The animals were killed by concussion and exsanguination. The glands were wrapped in Parafilm and placed in a deep freeze for at least 24 hours. The block was attached with a drop of buffer to a microtome chuck that had been precooled on CO_2 ice; very small organs were mounted on a piece of liver. The blocks were sectioned at 15 μ in a

[3] ACTHAR Gel, obtained from the Armour Laboratories through the courtesy of Drs. James Lesh and Joseph D. Fisher.

TABLE 2. COMPOSITION OF MEDIUM FOR DEMONSTRATING 3β-OL DEHYDROGENASE

Constituent	Amount	Final molarity
DHA[1]	0.2 mg.	ca. 1 mM
Propylene glycol	0.5 ml.	1 M
Nitro-BT[2] soln., 1 mg./ml.	1.0 ml.	0.16 mM
Nicotinamide[3] soln., 1.6 mg./ml.	0.7 ml.	4 mM
DPN[4] soln., 3 mg./ml.	0.8 ml.	0.54 mM
Phosphate buffer, 0.1 M, pH 7.1–7.4	4.0 ml.	0.057 M
	7.0 ml.	

[1] One ml. of acetone containing 0.2 mg. of DHA was put in a dry Columbia jar and the acetone was evaporated off in a 37° C oven. The other constituents were then added. The propylene glycol in the medium facilitates dissolving the substrate, which is present in slight excess of its solubility.

[2] 2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)ditetrazolium chloride; Dajac Laboratories, Philadelphia.

[3] Eastman Organic Chemicals, Rochester.

[4] Sigma Chemical Co., St. Louis.

Coons cryostat (9) (temperature, $< -20°$ C). Serial sections were mounted on consecutive #1 coverslips. These were either incubated immediately after a brief period of drying to attach the section to the slide, or stored in a deep freeze for later use.

In our standard incubations, sections were first placed for 5 minutes in 0.1 M phosphate buffer, pH 7.1–7.4, at room temperature to remove endogenous substrates. Some sections had been extracted with acetone for 5 minutes at 4° C to remove free lipide droplets. They were then incubated in a Columbia jar containing a dehydroepiandrosterone[4] (DHA) medium (Table 2) at 37° C for periods ranging from 5 minutes to 1 hour. (Incubations at room temperature were successful but necessarily longer.) Control sections were incubated in the same mixture lacking DHA. Other sections were incubated in a 50 mM sodium lactate medium resembling the DHA medium except that it lacked propylene glycol.

After incubation, the sections were fixed for 30 minutes in a mixture containing 50% ethanol and 10% formalin, then mounted with glycerol gelatin. For definite identification of tissue components, some sections were counterstained after fixation with Oil Red O for lipide droplets or with Grenacher's alum carmine for nuclei. In the study of the hypophysectomized rats and their controls, one adrenal and ovary and a piece of uterus were fixed in Carnoy's fluid; paraffin sections were stained with dilute eosin and methylene blue at pH 5 and by the Feulgen nucleal technique.

In preliminary tests, sections cut on the ordinary freezing microtome were incubated without prior drying. In a few runs, media buffered at higher pHs (7.8, 8.8) or containing differing amounts of the several constituents were used (see Results). Also tested were media containing Δ^5-pregnen-3β-ol-20-one (pregnenolone), testosterone, and androstan-17β-ol-3-one.

As reported by Nachlas *et al.* (10), Nitro-BT media may be used repeatedly. We routinely froze the media between experiments. Re-use about 5 times (for a total of 20–25 sections) was permissible. The principal reason for discarding a medium was the development of nonspecific staining, probably because of accumulation of endogenous substrates, rather than because of depletion of specific substrate, DPN or Nitro-BT.

RESULTS

Activity towards DHA in normal rat tissues. In untreated sections, formazan was deposited abundantly in all of the presumed steroid-secreting

[4] Obtained from General Mills Co., through the courtesy of Dr. A. D. Odell.

cells in rat adrenal, testis and ovary (Plate I). Adrenocortical cells were highly active, including those occurring in extraglandular nodules and those scattered in the medulla (Fig. 1). Testicular Leydig cells were also reactive (Fig. 2). In the ovary, the interstitial cells, lutein cells, and follicular thecal cells consistently displayed formazan deposits (Figs. 3–5). Granulosa cells were inactive in healthy follicles, but whenever free lipide droplets had developed in these cells (a sign of atresia (11)), moderate activity toward DHA also existed. Activity appeared only moderate in developing corpora lutea, very intense in large, fully-developed ones, and less marked in those undergoing involution (large lipide droplets). Within these organs, no other cell type developed any formazan deposit unless it also appeared in the control preparations, suggesting that this depended on the accumulation of endogenous substrates. Particularly prone to such nonspecific staining were the tails of spermatozoa within the seminiferous tubules (Fig. 2).

The relative degrees of reactivity after a given period of incubation were as follows: ovarian interstitial cells > adrenocortical cells, lutein cells in ripe corpora lutea, theca interna cells > Leydig cells > granulosa cells in atretic follicles. Activity was grossly visible within about 1 minute; accumulations of formazan adequate for microscopic study required 15–30 minutes, in weakly-active tissues, 1 hour.

When the sections were pretreated with cold acetone to remove the free lipide droplets, formazan deposition was only slightly reduced in all the above-mentioned cell types except in the follicular theca interna, which became virtually inactive (cf. Figs. 3, 4). Such preparations allowed a finer deposition of the formazan (4), since crystallization did not occur on the surfaces of the lipide droplets (10).

No activity toward DHA was detectable in kidney or liver sections with incubations lasting 1 hour. One cell type not implicated in steroid synthesis, namely, the ampullary epithelial cell of the oviduct, showed significant formazan deposit (see Fig. 12).

All activity was destroyed by pretreating the sections for 5 minutes with buffer at 80° C. Such inactivation occurred even in sperm tails, indicating that enzymatic activity, not oxidation of -SH groups, accounted for their staining. Considerable inhibition of activity occurred with as little as 5 minutes' pretreatment with Baker's calcium-formalin; severe inhibition occurred with pretreatment of sections for 1 hour or with overnight fixation of blocks.

Comparisons showed that enzymatic activity was not detectably diminished in deep-frozen blocks for over 2 weeks, if they were protected from dehydration. Also, dried sections kept in the deep freeze retained their activity for about 2 weeks. These facts permitted comparison of the activities of different organs under different conditions. Formazan deposit of course developed more quickly and more abundantly in free-floating sections that

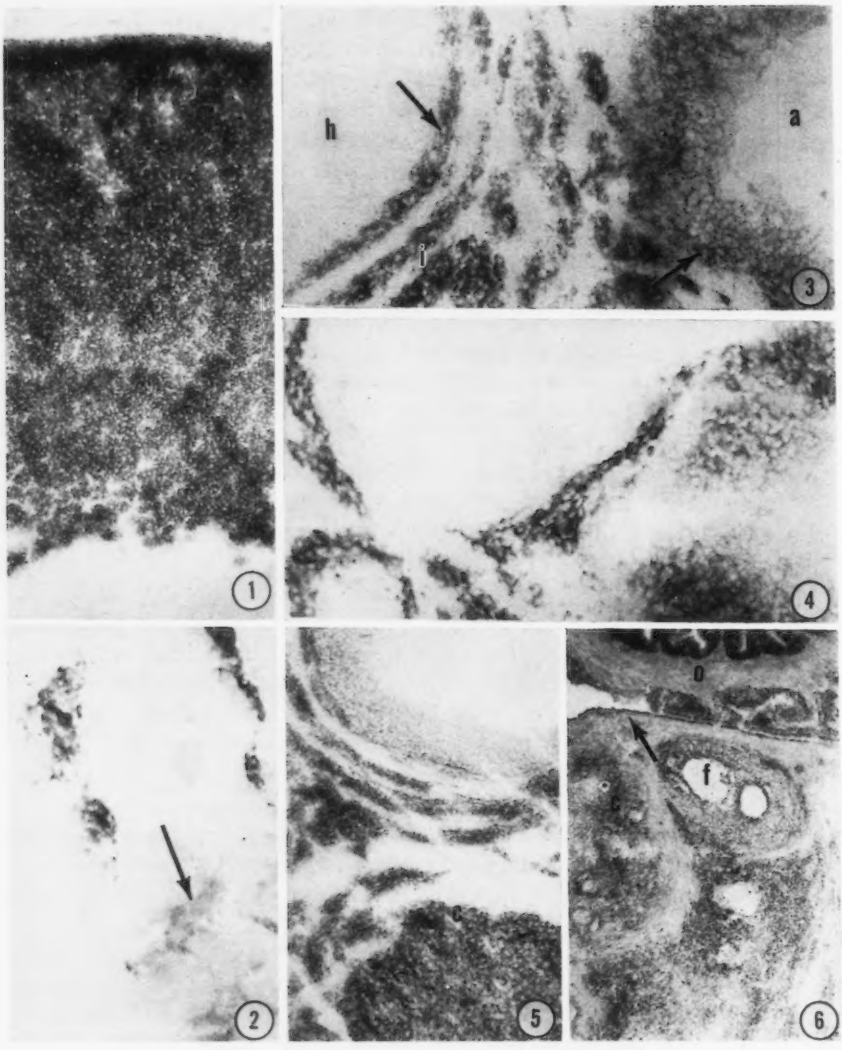

PLATE I. Dehydrogenase activity in normal rat organs.

FIG. 1. Cryostat section of an adrenal gland of a normal female rat; the section was pretreated with cold acetone, incubated for 1 hour in the DHA medium, ×69. The formazan deposit is limited to adrenocortical cells (cf. Fig. 9, incubated with lactate). The high activity of the glomerulosa illustrated here was not consistent.

FIG. 2. Section of a normal rat testis, pretreated with cold acetone, incubated for 1 hour with DHA, ×130. Activity is intense in the Leydig cells. The apparent activity of the spermatozoa (arrow) is nonspecific, i.e., it occurred in control sections incubated in a medium lacking substrate.

FIGS. 3 & 4. Nearby sections of an ovary of a normal rat in proestrus, incubated

had not been subjected to drying (12), but these were unsatisfactory from a morphological point of view.

Our medium for demonstrating steroid-3β-ol-dehydrogenase activity differs from Wattenberg's (4) in several respects. (a) It contains propylene glycol rather than acetone to solubilize the substrate. Pretreatment with or inclusion of acetone appeared to depress overall enzymatic activity slightly and greatly reduced the staining of the theca interna. (b) It contains less Nitro-BT. Increased concentrations of the particular batch of Nitro-BT used produced no benefit. In general, tetrazolium salts are themselves toxic and may also contain stabilizers that are inhibitory (12), so that the concentration should be no more than that allowing maximum hydrogen ion trapping. (c) It contains less DPN. A comparative study of media containing 0.27, 0.54 and 1.08 mM DPN showed no benefit in using more than 0.54 mM. The nicotinamide in the solution inhibits destruction of the DPN, as was indicated by results with re-used media. (d) It contains only phosphate buffer instead of the Krebs mixture. We did not test whether this factor affects enzymatic activity. (e) The pH is about 7.2 rather than 8.0. It is best to keep the pH as low as possible to prevent nonenzymatic reduction of the tetrazole by -SH groups (12). Also, with the glycol-containing medium, nonspecific activity occurred in all tissues when the pH was raised to 7.8 (4) or 8.8 (13); this, however, is not evident in Wattenberg's (4) illustrations. Apparently propylene glycol can be utilized as a substrate by widely-distributed dehydrogenases.

Activity toward other sterols. As reported by Wattenberg (4) and Pearson and Grose (13), the same tissues that oxidized DHA also oxidized another Δ^5-3β-hydroxysteroid, pregnenolone (Fig. 11). The amount of formazan deposited was always very much less with pregnenolone as substrate than with DHA. This difference may result from the poor solubility of pregnenolone.

With our media, a similarly low degree of activity was exhibited by these same tissues toward two 17β-hydroxysteroids, testosterone and androstan-

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with DHA, ×180. Fig. 3 incubated for 30 minutes without pretreatment; Fig. 4 first extracted with cold acetone, then incubated for 1 hour. The follicle at the left is healthy (h), that on the right is atretic (a); i, interstitial tissue. In Fig. 3, the thecal (arrows), interstitial and atretic granulosal cells react; in Fig. 4 the follicular thecal cells are unreactive.

FIG. 5. Section of a normal rat ovary incubated for 30 minutes with DHA, ×86. Strong reactivity of corpus luteum (c) and interstitial tissue; weaker reaction in the theca interna of the follicle (atretic), slight reaction in the granulosa of this follicle.

FIG. 6. Section of a normal rat ovary and oviduct, incubated for 45 minutes with lactate, ×86. Strong reactivity in oviduct (o) epithelium and "germinal" epithelium (arrow) and some of the granulosa cells in a small vesicular follicle (f); corpus luteum (c) and interstitial tissue moderately reactive, connective tissue and muscle slightly reactive.

17β-ol-3-one. Ovarian and testicular steroid-producing cells were more active than were adrenocortical cells. (With DHA and pregnenolone, adrenocortical tissue exhibited the same degree of activity as corpora lutea.) Previous workers have not observed staining with 17β-hydroxysteroids (4, 13). With our glycol-containing medium, no activity toward these substrates was evident (i.e., greater than in control sections) when the pH was raised to 7.8 or 8.8. Some activity toward the 17β-hydroxysteroids also occurred within hepatic and kidney tubular cells.

Activity toward lactate. With sodium lactate as substrate, formazan deposition occurred in all cell types except erythrocytes, although the quantity varied greatly from cell type to cell type (see Figs. 6, 9, 13, 15). In adrenals, activity was greatest in the periadrenal adipose-tissue cells and in the muscularis of arterioles; next in intensity was the cortical parenchyma, weakest were the medullary cells. Adrenocortical activity was apparently greater toward lactate than toward DHA, since a shorter incubation period was required to produce the same quantity of formazan (cf. Figs. 8, 9). In testis, the spermatozoa, the epithelium of the seminiferous tubules, and the interstitial cells were all highly active. Again, the Leydig cells appeared more active than in DHA preparations. In ovary and oviduct, activity was ubiquitous but somewhat less intense than with DHA in the steroid-producing cells (cf. Figs. 5, 6). Especially active were oviduct epithelium and the "germinal" epithelium (Figs. 6, 13). Formazan deposition was also conspicuous within the liver and kidney, in blood vessel walls as well as in parenchymal cells.

Unlike 3β-ol-dehydrogenase activity, lactic dehydrogenase activity appeared enhanced after treatment for 5 minutes with cold acetone and after treatment for either 5 or 60 minutes with cold calcium-formalin. Since this dehydrogenase is quite soluble in many tissues (14), the increased staining may have reflected fixation of the enzyme within the tissue. (Steroid-3β-ol dehydrogenase, on the other hand, appears to be bound to cellular structures, probably "microsomes" (15, 16).)

Responses of rat adrenal and ovary to hypophysectomy. During the shrinkage of the adrenals after hypophysectomy (Table 1), the inner zones of the adrenal cortex underwent progressive atrophy. By 7 to 10 weeks, all residual cells in this region appeared markedly acidophilic in eosin and methylene blue preparations. The cells in the glomerulosa and subglomerulosa remained healthy looking and moderately basophilic. Activity toward DHA and the other sterols tested appeared normal or somewhat enhanced in the outer portions of the adrenal cortex (zona glomerulosa and the lipide-free subglomerulosal zone) (Plate II). In the 3-day specimen, there was no detectable reduction of activity in the involuting inner cortex. Thereafter, however, activity gradually declined (Fig. 7), and by 50 days almost all activity had disappeared from the inner half of the cortex (Fig. 8). Activity toward lactate followed much the same pattern, but even at 50 days con-

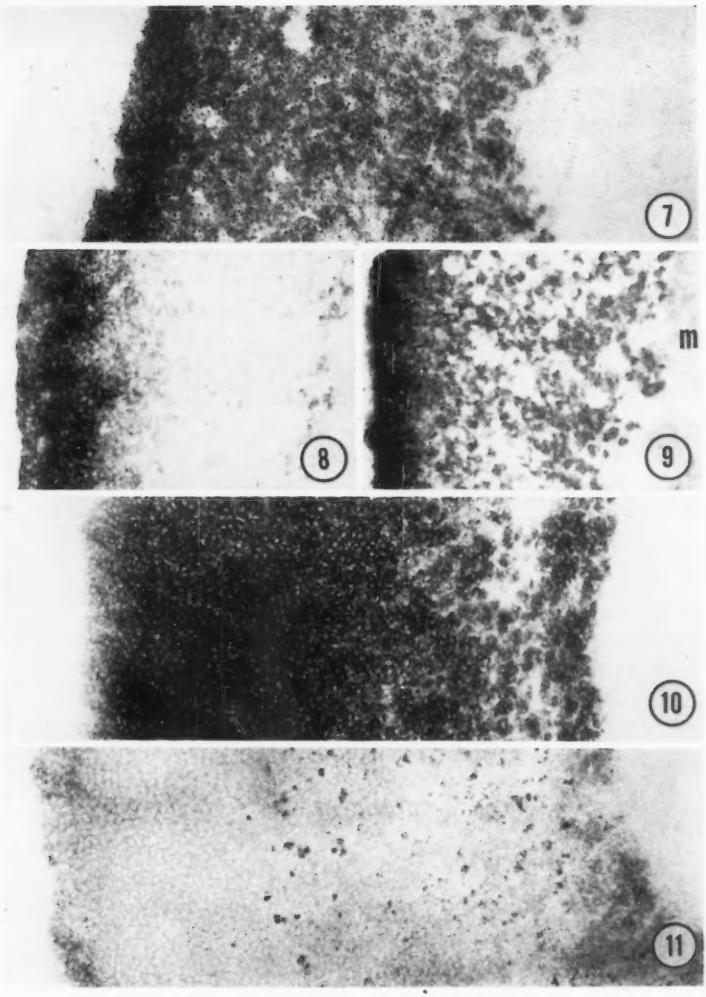

PLATE II. Dehydrogenase activity in adrenals of hypophysectomized female rats. All ×100.

FIG. 7. Adrenal of rat operated on 2 weeks earlier; section incubated for 45 minutes with DHA. The outer cortical zone is very reactive, the inner zones are less reactive than normal (cf. Fig. 1).

FIGS. 8 & 9. Adrenal sections of rat hypophysectomized 7 weeks earlier. Fig. 8, pretreated with cold acetone, incubated for 40 minutes with DHA; Fig. 9, pretreated with cold acetone, incubated for 15 minutes with lactate. The atrophied inner zones have lost virtually all activity toward DHA but not toward lactate. Note the moerate reactivity of medulla (m) toward lactate.

FIGS. 10 & 11. Adrenal sections of rat hypophysectomized 11 weeks earlier and treated for the last 8 days with ACTH. Fig. 10, pretreated with cold acetone, incubated for 50 minutes with DHA; Fig. 11, pretreated with cold acetone, incubated for 1 hour with pregnenolone. The activity of the hypertrophied inner cortex has been restored.

siderable activity toward this substrate remained in many cells of the inner zones (Fig. 9).

As the adrenals of animals hypophysectomized 70 days earlier enlarged under the stimulus of ACTH, the zone of reactive cortical cells broadened. After 8 days of treatment, all cortical cells displayed considerable activity (Figs. 10, 11).

The ovaries of the hypophysectomized rats showed the typical disappearance of vesicular follicles, regression of interstitial tissue, but maintenance of the corpora lutea. Small follicles lacked a differentiated theca. At progressive periods after operation, the enzymatic activity toward DHA and other hydroxysteroids gradually declined in the atrophying interstitial tissue (Plate III, Fig. 14). No decline was detectable in the corpora lutea, however, even as late as 11 weeks after the operation (ACTH-treated specimen). Formazan deposition persisted in the epithelium of the oviduct (Fig. 12), though perhaps somewhat reduced from normal. As in the adrenal gland, activity toward lactate was present in all residual cells, even the interstitial tissue (Figs. 13, 15).

DISCUSSION

The steroid hormone-producing cells in rat adrenal, testis and ovary possess activity toward 3β-hydroxysteroids, though the apparent levels differ. This limitation to a few cell types is in contrast to activity toward lactate, which occurs in all cell types except erythrocytes. Indeed, the level of activity toward lactate varies in comparison to activity toward 3β-hydroxysteroids in the different steroid-producing cells.

Samuels *et al.* (1) and Wattenberg (4) have provided chemical and histological evidence, respectively, for dehydrogenation of 3β-hydroxysteroids in all of these tissues except the theca interna of ovarian follicles. In our hands, the latter activity is severely reduced after treatment of the sections with acetone, a constituent of Wattenberg's medium. Such a solvent was not present in the Samuels medium, however. Whether or not this disappearance of formazan deposition in the theca reflects a special lability of the responsible thecal enzyme remains to be examined. That the presence of Nitro-BT formazan in this cell type bespeaks its migration to the lipides there appears unlikely from the uniformity of reaction and the general indications of local precipitation of this highly insoluble product (see also (4, 7, 10)). And the theca interna does not have an unusually high concentration of DPNH-tetrazolium reductase, to judge from the lactic dehydrogenase preparations.

All functional steroid hormones other than the estrogens possess a Δ^4-3-keto configuration, and in adrenocortical, testicular and corpus luteal tissues cholesterol and other Δ^5-3β-hydroxy compounds have been shown to be hormone precursors (1, 4). There is some evidence that estrogens are derivatives of Δ^5-3β-hydroxy and Δ^4-3-keto-C_{19} precursors (16, 17,

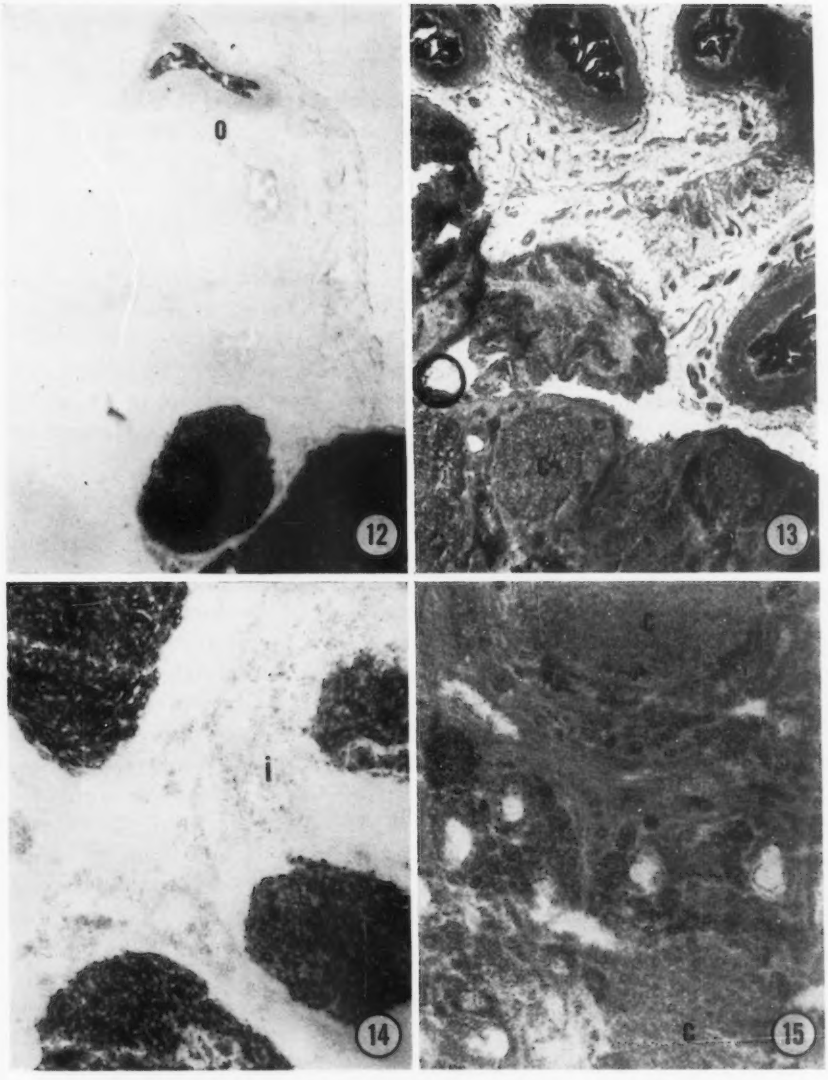

PLATE III. Dehydrogenase activity in ovaries of hypophysectomized rats.

FIGS. 12 & 13. Sections of ovary and oviduct of a rat hypophysectomized 7 weeks earlier, ×45. Fig. 12, incubated for 1 hour with DHA: the corpora lutea in the ovary (below) and the epithelium of the ampulla of the oviduct (o) react. Fig. 13, incubated for 1 hour with lactate: the oviduct epithelium and its muscle plus the blood vessels are highly reactive, the residual tissues within the ovary are normally reactive (cf. Fig. 6). c, Corpus lutuem.

FIGS. 14 & 15. Ovarian sections of long-term hypophysectomized rats, ×80. Fig. 14, incubated for 1 hour with DHA; Fig. 15, pretreated with cold acetone, incubated for 1 hour with lactate. With DHA, the corpora lutea have remained intensely reactive, but the interstitial tissue (i) is only weakly active. With lactate, reactivity of the corpora lutea (c) and interstitial tissue is essentially normal. (Cf. adrenal cortex, Figs. 8 & 9.)

18). Therefore the presence of the enzyme in theca interna cells, the presumed source of estrogens (11, 19), as well as in adrenocortical, Leydig, and lutein cells, is compatible with these biochemical observations. The activity in the granulosal layer of atretic follicles may indicate abortive luteinization (11). Just what hormone(s) the thecal cells residing in the interstitial tissue secrete remains unknown, but the origin of these cells makes their production of estrogen most likely.

The significance of the apparent activity of the oviduct epithelium remains to be determined. Two possibilities come to mind: It might signify a broad-spectrum alcohol dehydrogenase capable of attacking a Δ^5-3β-hydroxysteroid, or it might indicate a local metabolism of steroid hormones. Particularly in the hamster, we have found evidence of the ability of several nonendocrine tissues to oxidize DHA (8) as well as other hydroxysteroids.

Following removal of the pituitary, the activity of residual endocrine cells in ovary and adrenal at first fails to undergo any striking diminution. Only as the interstitial tissue and the cells of the inner zones of the adrenal cortex become severely atrophied does their enzyme activity greatly decline (1–2 months). That these cells are not yet moribund, however, is indicated by their retention of lactic dehydrogenase activity. The corpora lutea and both the glomerulosa and the subglomerulosal zone of the adrenal cortex, all of which remain healthy-looking, retain their activity toward DHA. The presence of this enzyme therefore appears to indicate a genetic capacity to form steroid hormones; it does not necessarily depend on stimulation or signify continued secretory activity. Of the tissues listed, only the zona glomerulosa has been shown to be secretory after hypophysectomy (20). It is noteworthy that Samuels and Helmreich (2) found no great change in the concentration of this enzyme in either testis or adrenal gland following hypophysectomy.

With our medium, dehydrogenation of 17β-hydroxysteroids is also detectable in the steroid-producing cells, as well as in liver, kidney and oviduct epithelium. Wattenberg (4) and Pearson and Grose (13) observed no activity toward such substrates in the steroid-producing glands, though the latter authors reported oxidation by liver cells. Just what difference in the three methods explains this discrepancy will require further study. Biochemically, the capacity of steroid-producing and many other tissues to oxidize such 17β-hydroxysteroids as estradiol and testosterone is established (21).

Acknowledgment

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REFERENCES

1. Samuels, L. T., M. L. Helmreich, M. B. Lasater and H. Reich: *Science* **113:** 490. 1951.
2. Samuels, L. T. and M. L. Helmreich: *Endocrinology* **58:** 435. 1956.
3. Rubin, B. L. and R. I. Dorfman: *Endocrinology* **61:** 601. 1957.
4. Wattenberg, L. W.: *J. Histochem. and Cytochem.* **6:** 225. 1958.
5. Farber, E., W. H. Sternberg and C. E. Dunlap: *J. Histochem. and Cytochem.* **4:** 254. 1956.
6. Novikoff, A. B.: *In* Analytical Cytology, 2nd Ed., R. C. Mellors, Ed., Chap. 2, McGraw-Hill Co., New York, 1959.
7. Cascarano, J. and B. W. Zweifach: *J. Biophys. and Biochem. Cytol.* **5:** 309. 1959.
8. Levy, H., H. W. Deane and B. L. Rubin: *J. Histochem. and Cytochem.* **7:** 320. 1959. (Abstract)
9. Coons, A. H., E. H. Leduc and M. H. Kaplan: *J. Exp. Med.* **93:** 173. 1951.
10. Nachlas, M. M., K.-C. Tsou, E. de Souza, C.-S. Cheng and A. M. Seligman: *J. Histochem. and Cytochem.* **7:** 420. 1957.
11. Deane, H. W.: *Am. J. Anat.* **91:** 363. 1952.
12. Deane, H. W., R. J. Barrnett and A. M. Seligman: *In* Handbuch der Histochemie, W. Graumann and K. Neumann, Eds., Vol. VII/1, Gustav Fischer-Verlag, Stuttgart, Germany, 1959.
13. Pearson, B. and F. Grose: *Fed. Proc.* **18:** 499. 1959. (Abstract)
14. Novikoff, A. B. and M. Arase: *J. Histochem. and Cytochem.* **6:** 397. 1958. (Abstract)
15. Beyer, K. F. and L. T. Samuels: *J. Biol. Chem.* **219:** 69. 1956.
16. Ryan, K. J.: *J. Biol. Chem.* **234:** 268. 1959.
17. Ryan, K. J.: *Endocrinology* **63:** 392. 1958.
18. Baggett, B., L. L. Engel, L. Balderas, G. Lanman, K. Savard and R. I. Dorfman: *Endocrinology* **64:** 600. 1959.
19. Deane, H. W. and A. M. Seligman: *Vitamins and Hormones* **11:** 173. 1953.
20. Rauschkolb, E. W., G. L. Farrell and S. Koletsky: *Am. J. Physiol.* **184:** 55. 1956.
21. Talalay, P.: *Physiol. Revs.* **37:** 362. 1957.

A COMPARATIVE IMMUNOLOGICAL STUDY OF PITUITARY GROWTH HORMONE FROM VARIOUS SPECIES

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ABSTRACT

Similarities and differences in the antigenic structures of pituitary growth hormone (somatotropin, STH) from various species were studied by means of anaphylactic shock experiments in guinea pigs, precipitin ring tests and agar gel-diffusion studies with antisera to highly purified bovine and human STH. Antihormone tests in hypophysectomized rats were also performed, with the same antisera.

The results of the above studies have suggested that the antigenic structures of pituitary STH from man and monkeys are very similar to one another, but are clearly different from those of the whale, hog, sheep, ox, horse and fish; and, furthermore, that the antigenic structures of sheep and ox STH's are closely related, but quite different from STH of all other species tested.

It was also demonstrated that antiserum to either the bovine or human purified STH could not only detect minute amounts of the homologous purified STH in saline solution and neutralize its biological activity, but could also detect STH in a crude pituitary extract and significantly neutralize the growth-promoting effect of such an extract. These results have suggested that the antibodies to the purified STH may also be specific for STH as it exists in the crude state. This was further supported by the findings from the agar gel-diffusion studies which revealed the presence of a substance in the crude extracts that was antigenically indistinguishable from the homologous, purified STH. It is felt, therefore, that the similarities and differences noted above in the STH's from the various species are real, and not the result of any artifact arising from the physicochemical manipulations required for the purification of the hormones employed in the present investigation.

IT IS known that various physicochemical differences exist between the pituitary growth hormone of bovine origin on the one hand and that of man on the other. For example, they have been shown to differ considerably with respect to their general structural configurations, molecular weights and isoelectric points (1, 2, 3). Moreover, it has also been demonstrated that monkey growth hormone is similar to human growth hormone (1), and sheep growth hormone to the bovine hormone (4), with respect to several of their properties. On the basis of the above observations, and since good antisera to bovine growth hormone (5, 6) as well as to the

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human hormone (7) were available, an investigation was undertaken to study and compare the purified pituitary growth hormone preparations from these and other species by means of immunological procedures.

MATERIALS AND METHODS

Antiserum

The antiserum to bovine pituitary growth hormone (somatotropin or STH) was prepared by the immunization of rabbits as previously outlined (6). The hormone in the form of a fine powder was suspended in the adjuvant, Bayol-Arlacel (8), without the customary aqueous phase. Young albino rabbits were injected subcutaneously once a week over a period of several months, with a total dose of approximately 80 mg. of the hormone per rabbit. The antiserum to human growth hormone was prepared by immunization of similar rabbits (7) with the hormone in Freund's adjuvant (Difco). The animals were injected initially with a total of 4 mg. of the hormone, 2 mg. by subcutaneous route and 2.0 mg. by intraperitoneal route. Two weeks later this procedure was repeated. After another interval of two weeks, each animal was injected with 2 mg. of the hormone in the form of an alum precipitate, 1 mg. subcutaneously and 1 mg. intraperitoneally, bringing the total dose of STH per rabbit to 10.0 mg. The rabbits were bled by cardiac puncture 10 days later. The antisera to both bovine and human STH were stored frozen, or were preserved with merthiolate in a final concentration of 1 to 10,000 and kept under refrigeration (2–5° C). Before the antisera were used, certain nonspecific antibodies were removed by absorption (6, 7).

Hormones

The growth hormones from both bovine and human sources were highly purified preparations which have been *characterized* previously (9, 3). The sheep (4), hog (10), whale (11) and monkey (3) hormones were also highly purified preparations.

Animals

Young albino rabbits, weighing approximately 2.5.to 2.8 kilograms, were employed for the production of antisera to bovine and human growth hormone. Growth hormone activity was assayed according to the tibia test (12) in hypophysectomized rats of the Long-Evans strain. Young female guinea pigs, weighing 375 to 450 gm., were used in all anaphylactic shock experiments. The respective diets given these experimental animals have been previously described (6).

General Methods

The specificity of the growth hormone antigen-antibody reaction was studied by means of four different techniques; namely, precipitin ring tests, precipitin tests in agar, anaphylactic shock experiments in guinea pigs and antihormone tests in hypophysectomized rats with antisera to STH. The Ouchterlony technique (13, 14) was employed for the precipitin tests in agar. General operational details of the other techniques have been described elsewhere (6, 7).

EXPERIMENTAL AND RESULTS

Precipitin Ring Tests

For the precipitin ring test, serial dilutions, in saline, of highly purified pituitary growth hormones from various species, including sheep, hog, whale, monkey, and man, were prepared, with 0.5 cc. aliquots of hormone

TABLE 1. REACTION OF STH FROM VARIOUS SPECIES WITH RABBIT ANTISERUM TO BOVINE STH, ACCORDING TO PRECIPITIN RING TESTS

	Reaction										
STH	500	250	125	63	32	16	8	4	2	1	0.5
	μg.										
Bovine	+	+	+	+	+	+	+	+	+	+	0
Ovine	+	+	+	+	+	+	+	+	+	0	0
Whale	+	±	0	0	0	0	0	0	0	0	0
Porcine	0	0	0	0	0	0	0	0	0	0	0
Monkey	±	0	0	0	0	0	0	0	0	0	0
Human	0	0	0	0	0	0	0	0	0	0	0

for each dilution. 0.1 cc. of the absorbed STH antiserum was then carefully layered under the hormone in each tube. The results are presented in Table 1. It may be observed that with bovine STH the highest dilution which gave a positive test contained 1 μg. of antigen, whereas ovine STH gave a positive test at a dilution of 2 μg., and hog STH was negative even at 500 μg. These results suggested that bovine STH was antigenically similar to ovine STH, but quite different from hog STH. The growth hormones from the whale, monkey and man were also found to differ considerably from the bovine STH in this respect.

Antiserum to human STH was then tested in a similar manner with STH from the monkey, whale, and bovine sources (Table 2). It is notable that the antiserum to human STH was capable of detecting as low as 0.25 μg. of hormone from the same species, and furthermore that it cross-reacted with monkey STH to the same degree as with the human hormone. This indicated that the hormones from these two primate species are very closely related antigenically. On the other hand, the human antiserum gave no positive reaction with either whale or bovine STH even at dose levels of 500 μg.

Precipitin Tests in Agar

The wells in the agar were made by placing a suitable brass mold in a petri dish, then pouring the desired amount of melted agar (1% in 0.85% saline) around the mold, and carefully removing the mold after the agar had cooled and solidified. Twenty-five to 30 cc. of agar were usually poured

TABLE 2. REACTION OF STH FROM VARIOUS SPECIES WITH RABBIT ANTISERUM TO HUMAN STH, ACCORDING TO PRECIPITIN RING TESTS

	Reaction										
STH	500	250	125	32	8	4	2	1	0.5	0.25	0.12
	μg.										
Human	+	+	+	+	+	+	+	+	+	+	0
Monkey	+	+	+	+	+	+	+	+	+	+	0
Bovine	0	0	0	0	0	0	0	0	0	0	0
Whale	0	0	0	0	0	0	0	0	0	0	0

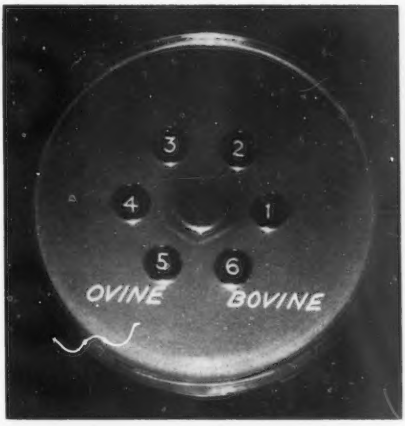

FIG. 1. The interaction of antiserum to bovine STH with purified STH preparations of various species, as determined by the Ouchterlony test. Central well contains antiserum to bovine pituitary STH; wells 1–6 contain 10 μg. each of human, monkey, porcine, whale, ovine and bovine STH, respectively.

into each petri dish. The agar was preserved with 1:10,000 merthiolate and was buffered at pH 7.6–7.8 with Tris (hydroxymethyl) aminomethane (Sigma and Co., St. Louis, Mo.).

In an agar plate, 0.3 cc. of rabbit antiserum to bovine STH was placed in a central well which was surrounded by six other wells each of which contained 10 μg. of STH from human, monkey, porcine, whale, ovine and bovine sources in 0.2 cc. of saline (Fig. 1). The arrangement of the second agar plate (Fig. 2) was the same as the first, except that the central well contained antiserum to human STH instead of antiserum to bovine STH. The plates were incubated at room temperature (20–22°C) and were photographed after 44 hours of incubation.

In the first plate, in which antiserum to bovine STH was employed (Fig. 1), a single precipitation line occurred between the central well and the well containing bovine STH, and also between the central well and the ovine

FIG. 2. The interaction of antiserum to human STH with purified STH preparations from various species, as determined by the Ouchterlony test. Central well contains antiserum to human pituitary STH; wells 1–6 contain 10 μg. each of human, monkey, porcine, whale, ovine and bovine STH, respectively.

STH wells. These 2 lines remained as single lines, suggesting a homogeneous antigen-antibody system in each instance; moreover the lines fused completely, and did not cross throughout 10 days of incubation, indicating that bovine and ovine STH are antigenically very closely related. Some differences were noted, however, in that the homologous bovine STH-antiserum system gave a precipitin reaction line that was slightly more intense and noticeably longer than the line between the bovine antiserum and ovine STH. It was of interest to note that the antiserum gave no reaction with any of the other STH preparations tested, including that of man.

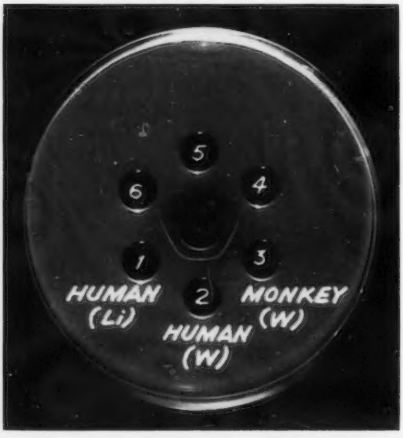

FIG. 3. The interaction of antiserum to human STH (Li) with STH's of various species prepared by Wilhelmi, as determined by the Ouchterlony test. Central well contains antiserum to human pituitary STH (Li); wells 1–6 contain 10 μg. each of the following growth hormone preparations: 1) human (Li), 2) human (Wilhelmi), 3) monkey (Wilhelmi), 4) porcine (Wilhelmi), 5) horse (Wilhelmi), 6) fish (Wilhelmi).

In the second plate (Fig. 2), the antiserum to human STH gave a sharp single, precipitin line with both human and monkey STH. Again, the two lines remained as single lines and maintained perfect fusion during the entire incubation period, indicating the extremely close antigenic relationship between human and monkey STH preparations. The precipitin reaction between the human STH and its antiserum, however, gave a line that was slightly more intense and longer than the line between the human antiserum and the monkey STH. Once more, there was a notable absence of any reaction of the antiserum with STH of other species tested.

Two more agar plates were set up in order to determine whether the antisera to the human and bovine STH preparations of Li (1, 15) would react with any of the STH samples prepared by Wilhelmi.[2] In the first of these plates (Fig. 3) antiserum to human STH (Li) was placed in the central well and allowed to react with 10 μg. each of STH from man, monkey, hog, fish and the horse (Wilhelmi); human STH of Li was used for a control. The antiserum gave positive precipitin reactions only with the

[2] The authors are very grateful to Dr. A. E. Wilhelmi for providing us with samples of his human, monkey, hog, fish and equine pituitary STH.

human and monkey STH preparations, which indicated that the primate STH preparations obtained by these two different investigators are antigenically very similar, if not identical; furthermore, the lack of reaction with STH from the hog, fish, and horse suggested the antigenic dissimilarity of the hormones of these species to the primate growth hormones, under the conditions of this experiment. It was interesting to note that the precipitation line obtained with Wilhelmi's human STH fused with the line obtained with his monkey hormone and with that obtained with the human STH of Li. The fusion of all three lines as one continuous line indicated the antigenic identity of these STH preparations.

In the fourth plate, the same test was repeated with the STH preparations of Wilhelmi, except for the substitution of antiserum to human STH with antiserum to the bovine STH of Li. All tests were negative, except for the control reaction line with the bovine STH of Li. These results indicated that bovine STH differs antigenically from the STH of all other species tested, including man, monkey, hog, horse, and fish.

Anaphylactic Shock Experiments

Forty young female guinea pigs were sensitized with human STH by the same procedure previously reported for bovine STH (6). In brief, the hormone was ground to a fine powder and was suspended in the adjuvant Bayol-Arlacel (2:3 parts). The animals were sensitized with two subcutaneous injections (0.4 cc. each) of the hormone suspension, administered three days apart, and were challenged 18–21 days after the first injection with STH from various species by intracardial injection of the hormone in 1 cc. of saline solution. The results of this experiment are presented in Table 3.

The human STH preparation was found to be a good antigen for the sensitization of guinea pigs. The majority of the animals that had been sensitized with the 0.1 mg. dose of the hormone succumbed in a typical fashion when they were injected intracardially with .025 to .200 mg. of the same hormone in saline solution. Another group sensitized to human STH in a similar manner was challenged with .050 to .200 mg. of monkey STH. Again, the majority of the animals died. However, when the animals sensitized with the human STH were challenged with similar and higher doses of either whale or bovine STH, not a single one of the ten injected animals died. It was further demonstrated that the sensitizing dose could be lowered to .010 mg. with equally effective results.

Antihormone Tests with Bovine STH Antiserum

In the present investigation, the precipitin ring tests and precipitin tests in agar as well as the anaphylactic shock experiments have suggested the species specificity of the bovine STH-antibody reaction. It was previously demonstrated that antiserum to bovine STH could neutralize the biological

activity of bovine STH (5, 6) and that the antiserum to human STH could neutralize the activity of human STH (7). Now, it was deemed desirable to further test this specificity by an antihormone test; i.e., by determining whether or not the antibodies formed against bovine STH could neutralize the biological activity of human STH. Thirty-two female rats of the Long-Evans strain were hypophysectomized at twenty-eight days of age and used two weeks postoperatively for the standard four-day tibia assay (12) to determine STH activity. These experimental animals were apportioned into six groups, one of which served as saline-treated controls. A second

TABLE 3. ANAPHYLACTIC SHOCK REACTIONS OF GUINEA PIGS SENSITIZED TO HUMAN STH AND CHALLENGED WITH STH FROM VARIOUS SPECIES

Sensitizing dose of human STH	Challenge			
	Agent (STH)	Dose	Reaction*	
mg.		mg.		
.100	Human	.200	3/3	8/10
		.100	3/4	
		.025	2/3	
.100	Monkey	.200	2/3	6/9
		.100	2/3	
		.050	2/3	
.100	Whale	.200	0/3	0/6
		1.000	0/3	
.100	Bovine	.200	0/1	0/4
		.300	0/1	
		.500	0/1	
		1.000	0/1	
.010	Human	.200	2/3	8/11
		.100	2/2	
		.050	2/3	
		.025	2/3	

* Typical severe reactions followed by death within 3–5 minutes.

group received antiserum to bovine STH, and the third and fourth groups received 10 μg. daily of bovine STH and human STH, respectively. The fifth and sixth groups also received bovine and human STH, respectively, but were injected in addition with antiserum to bovine STH. The antiserum was absorbed free of certain nonspecific antibodies (6) prior to use. The daily dosage of serum was 0.25 cc., injected intraperitoneally, whereas the STH was dissolved in 0.5 cc. of saline solution and injected subcutaneously in the dorsum. The animals were sacrificed on the fifth day, 22 to 24 hours after the last injection of hormone.

It may be seen from Table 4 that the administration of antiserum alone caused no significant change in the width of the tibial epiphyseal cartilage plate over those of the saline-injected controls. On the other hand, the injection of bovine or human STH alone elicited a highly significant increase in tibial plate width. When the antiserum to bovine STH was injected in

addition to the respective hormones, the activity of the bovine hormone was completely neutralized ($P < .001$) whereas the activity of the human STH was not influenced in the least. The species specificity of the bovine STH antibodies was thus clearly demonstrated, in support of the findings reported above with the precipitin tests and anaphylactic shock experiments.

Another antihormone experiment was carried out, which suggested that the antiserum to purified bovine pituitary STH could not significantly neutralize the rat's endogenous STH. Five normal female rats, 28 days of age, were injected subcutaneously daily for 9 days with increasing amounts of unabsorbed rabbit antiserum to bovine STH. The amount of antiserum injected in the first half of the experiment (0.5 to 1 cc. daily) was estimated to be sufficient to neutralize a level of STH equivalent to 50 to 100

TABLE 4. THE INFLUENCE OF RABBIT ANTISERUM TO BOVINE STH ON THE BIOLOGICAL ACTIVITY OF BOVINE AND HUMAN STH, DETERMINED BY THE STANDARD 4-DAY TIBIA TEST

Group*	No. of rats	Tibial plate width
		$\mu \pm$S.E.
I. Saline	6	161 ± 2.3
II. Antiserum	5	157 ± 3.6
III. Bovine STH	6	231 ± 7.0
IV. Human STH	4	229 ± 7.9
V. Bovine STH + A/S	6	171 ± 4.6
VI. Human STH + A/S	5	233 ± 8.3

* Dosage of bovine or human STH was 0.010 mg. daily for 4 days, and antiserum (A/S) dosage was 0.25 cc. daily.

μg. daily of purified bovine STH (6), and in the last half of the experiment (2 to 3 cc. daily) sufficient to neutralize 200 to 300 μg. daily. Despite this moderately high level of antiserum, no significant influence on the rate of body weight gain was demonstrable when compared with the rate of gain of 8 other similar rats treated with corresponding amounts of normal rabbit serum for the same period of time.

Identification of STH in a Crude Pituitary Extract by Serological and Bioassay Procedures

The results of the antihormone experiments reported above have furnished evidence in favor of the specificity of the bovine and human STH reactions with their homologous antibodies; however, the experiments were carried out only with purified growth hormone and its antiserum. Consequently, an experiment was designed to determine whether or not the antiserum to the purified STH would be specific for the hormone as it actually exists in its crude state, before it had undergone the physicochemical manipulations necessary for its purification.

A crude alkaline extract was prepared from the anterior lobe of a single

bovine pituitary gland. The lobe was a large one, weighing 1.6 gm., frozen weight. The tissue was homogenized and extracted in the cold in 250 cc. of water. After centrifugation, sufficient water was added to the extract to bring the final volume to 320 cc. The pH was adjusted to approximately 8.5 with 0.1 N HCl before the solution was used for the serological tests and for the bioassay.

0.5 cc. portions of this stock extract were tested for STH content by the precipitin ring test with absorbed antiserum to bovine STH. The highest dilution of the known control of purified bovine STH which gave a positive reaction with 0.1 cc. of the antiserum was 0.25 μg. The highest dilution (1:64) of the crude extract that gave a definite positive test was then as-

TABLE 5. INFLUENCE OF RABBIT ANTISERUM TO BOVINE STH UPON THE TIBIAL PLATE-STIMULATING EFFECT OF A CRUDE, BOVINE PITUITARY EXTRACT

Group*	Av. initial body weight	Av. change body weight	Tibial plate width†
	gm.	gm.	μ
Hypophysectomized Controls	71.7	−1.4	161 ± 2.3
I. NRS+P.E. (undiluted)	71.5	+6.7	270 ± 4.3
II. A/S+P.E. (undiluted)	71.0	+1.8	189 ± 4.1
III. NRS+P.E. (dil. 1:1)	71.2	+5.0	231 ± 5.3
IV. A/S+P.E. (dil. 1:1)	72.8	+1.3	160 ± 3.5

* 6 rats in each group. NRS = normal rabbit serum; A/S = antiserum to purified bovine STH; P.E. = crude extract of bovine pituitary anterior lobe.

† It has been previously shown (6) that a 1 cc. daily dose of normal rabbit serum alone, has no significant effect on the tibial response.

sumed to contain the same amount of bovine STH, 0.25 μg. With correction for the dilution, 0.5 cc. of the undiluted stock extract was estimated to contain .016 mg. of STH. On this basis, the 320 cc. total volume of the extract was calculated to contain 10.24 mg. of STH, a value which roughly represented the total STH content of this single pituitary gland.

Aliquots of the same crude extract were then tested for STH activity by the standard 4-day tibia test (12). Twenty-four hypophysectomized rats were apportioned into 4 uniform groups of 6 animals each on the basis of body weight. Two different dose levels were administered. Animals of groups I and II were all injected with 0.5 cc. of the stock extract and in addition received 0.5 cc. of either normal rabbit serum or STH antiserum, respectively (Table 5). The animals of groups III and IV were injected with one half this dosage of both pituitary extract and serum. All injections were given intraperitoneally.

The 0.5 cc. daily dose of the undiluted pituitary extract (group I) gave a tibial response of 270 μ, whereas the same volume of the extract diluted by one half (group III) gave a response of 231 μ. On the basis of the standard dose-response curve for purified STH, such responses would be obtained by daily doses of approximately .020 mg. and .010 mg., respectively, of the latter. By correcting for the dilution and original total volume of the ex-

tract, the 320 cc. of the extract was calculated to contain 12.8 mg. of STH, as compared to the 10.2 mg. obtained by serological estimation, representing in both instances the total STH content of a single pituitary gland. Thus the results obtained by these two methods were in general agreement, although the estimate by bioassay was somewhat higher than the estimate according to the serological procedure.

These results have suggested that with antiserum to the highly purified STH, one can not only detect STH but also obtain a rough estimate of the amount of STH contained in a crude pituitary extract.[3] Moreover, the

FIG. 4. Interaction of antiserum (A/S) to human growth hormone (HGH) with crude human pituitary extract (HPE), as determined by the Ouchterlony test. Top well contains 0.2 cc. of antiserum; lower right well contains 0.2 cc. of crude pituitary extract (obtained from a total volume of 320 cc., the total extract from a single pituitary gland).

tibial-stimulating effect of the crude extract, an effect which can be assumed to be due primarily to growth hormone, was also completely neutralized by the antiserum to the purified STH.

In order further to elucidate the question of the identity of purified pituitary STH with the hormone as it exists in the crude state, the agar gel-diffusion test was again employed. In this instance, the test was set up with triangular instead of circular wells. One well contained 0.3 cc. of rabbit antiserum to human STH; the other two wells contained the antigens, namely, 0.2 cc. of human STH (10 μg.) and 0.2 cc. of the crude pituitary extract, respectively (Fig. 4). The agar plate was incubated at room temperature for 1 week. It can be seen that a single line appeared between the antiserum well and each of the two antigen wells. The complete fusion of these two lines indicated that there is in the crude human pituitary extract a substance which is immunologically indistinguishable from the purified human growth hormone. When a parallel test in agar was performed, in

[3] It is not proposed, however, that the serial dilution technique be employed routinely for assay of STH in a pituitary extract or in biological fluids, owing to the relatively large errors which may occur with this technique. For qualitative determinations, however, the method is excellent, if properly controlled.

which the antiserum to purified bovine STH was allowed to react with bovine STH and a crude bovine pituitary extract, the results again showed the antigenic identity of purified bovine STH and a substance in the crude extract that is presumably bovine STH.

DISCUSSION

It has been of interest that growth is elicited in the rat by injections of pituitary STH from practically all the species studied so far, including human, monkey, hog, whale, sheep and ox, with the only exception being the hormone from fish glands (16). In view of the fact that there are considerable differences among these various growth hormones in structure and molecular weight (17, 18) it is remarkable that the rat is capable of such a wide response. On the other hand, although it has been demonstrated that man can respond to the growth-promoting effect of human (3, 18, 19, 20) or monkey (3, 19) STH, practically all attempts to obtain such a response in man with bovine STH have hitherto failed (21, 22). The question has been raised as to the possible existence of a common biologically active core (3) or nucleus (23) in the STH molecule from the various species, and as to whether the rat might possess the necessary enzyme systems to utilize this core whereas man does not (3).

The studies presented above represent an attempt to gain some insight into these problems through the use of immunological procedures. The results of these studies have demonstrated that antibodies to bovine STH cross-react almost completely with sheep STH but manifest no reaction with the human or monkey hormones, on the basis of precipitin tests and anaphylactic shock experiments. Thus, bovine and sheep STH appear to be closely related antigenically, whereas bovine STH and the primate hormones are very different in this respect. Antihormone experiments have given further evidence in support of the species specificity of the reactions involved in the above observations, by demonstrating that antibodies to bovine STH can completely neutralize the biological activity of bovine STH but not that of human STH.

Since the molecular weight of human STH is relatively small, 27,000 (1, 3), as compared with 46,000 and 49,000, respectively, for bovine and ovine STH (9, 4), it occurred to us that antibodies to this hormone might cross-react with growth hormones, obtained from various other species, whose molecular weights are higher. This would be true particularly if the antibodies were actually directed against a biologically active core common to the growth hormones from the different species. We have observed previously that antibodies to human STH can completely neutralize human STH (7). In the present investigation, it was noted on the basis of the agar gel-diffusion tests that these antibodies were capable of almost complete cross-reaction with monkey STH. However, there was no evidence, on the basis of precipitin ring and agar gel-diffusion tests as well as

the antihormone and anaphylactic shock experiments, that these antibodies to human STH could react with STH from any of the other species tested. These findings would suggest that the antibodies we have obtained are not directed primarily against a common biologically active core, but, rather, against other portions of the molecular structure, portions which may be responsible for the species specificity. This does not preclude the possibility, however, that there may still be a common denominator in the molecular structures of the growth hormones from the various species. The specificity of the antibodies to STH may depend upon the over-all configuration of the STH molecule, including the biologically active portion, and not necessarily upon the structure of the latter alone.

One might raise the question as to whether or not the observed similarities and differences in the antigenic structure of pituitary STH from the various species could be due to an artifact. In other words, could the particular antigenic qualities of the purified hormones be the result of the various physicochemical manipulations required for the purification of the hormones? Can the results we have obtained with the purified hormones be applied to STH as it actually exists in the pituitary gland? It is felt that the parallel results obtained with the various types of tests employed in the present investigation lend support in favor of the antigenic identity of purified STH and the hormone as it exists in the pituitary gland. Probably the strongest support comes from the fact that the growth-promoting influence of a crude pituitary extract can be neutralized by antibodies to the purified growth hormone, and the fact that the agar gel-diffusion test revealed the presence of a substance in the crude extract which was immunologically indistinguishable from purified STH.

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REFERENCES

1. Li, C. H. and H. Papkoff: *Science* **124:** 1293. 1956.
2. Li, C. H.: *Cancer* **10:** 698. 1957.
3. Li, C. H.: *Fed. Proceedings* **16:** 776. 1957.
4. Papkoff, H. and C. H. Li: *Biochimica et Biophysica Acta* **29:** 145. 1958.
5. Hayashida, T. and C. H. Li: *Anatomical Record* **130:** 313 (Abstract #270). 1958.
6. Hayashida, T. and C. H. Li: *Endocrinology* **63:** 487. 1958.
7. Hayashida, T. and C. H. Li: *Science* **128:** 1276. 1958.
8. Cohn, M.: in *Methods in Medical Research* **5:** 275. Year Book Publishers. Chicago, 1952.
9. Li, C. H., H. Clauser, P. Fønss-Beck, A. L. Levy and H. Papkoff: in *Hypo-*

physeal Growth Hormone, Nature and Actions, edtied by Smith, R. W., Jr., O. H. Gaebler and C. N. H. Long. Blakiston, New York, 1955. P. 83.

10. Papkoff, H. and C. H. Li: unpublished data.
11. Papkoff, H. and C. H. Li: *J. Biol. Chem.* **231:** 367. 1958.
12. Greenspan, F. S., C. H. Li, M. E. Simpson and H. M. Evans: *Endocrinology* **45:** 455. 1949.
13. Ouchterlony, O.: *Acta Pathol. Microbiol. Scand.* **32:** 231. 1953.
14. Ouchterlony, O.: *Acta Pathol. Microbiol. Scand.* **26:** 507. 1949.
15. Li, C. H.: *J. Biol. Chem.* **211:** 555. 1954.
16. Wilhelmi, A. E.: Cited by G. E. Pickford in *Endocrinology* **55:** 274. 1954.
17. Li, C. H.: *Laboratory Investigation* **8:** 574. 1959.
18. Li, C. H.: in Symposium on Protein Structure, pp. 302 (1958), edited by A. Neuberger, Methuen, London.
19. Beck, J. C., E. E. McGarry, I. Dyrenfurth and E. H. Venning: *Science* **125:** 884. 1957.
20. Pearson, O. H., M. B. Lipsett, E. Greenberg and B. S. Ray: (Abstract #40). Program of 39th Meeting of the Endocrine Society, New York, N. Y. 1957.
21. Bennett, L. L., H. Weinberger, R. Escamilla, S. Margen C. H. Li and H. M. Evans: *J. Clin. Endocrinol.* **10:** 492. 1950.
22. Shorr, E., A. C. Carter, R. W. Smith, Jr., B. J. Kennedy, R. J. Havel, T. N. Roberts, L. L. Sonkin adn E. T. Livingstone: in *The Hypophyseal Growth Hormone, Nature and Actions,* edited by Smith, R. W., Jr., O. H. Gaebler, and C. N. H. Long. Blakiston, New York, 1955. P. 83.
23. Wilhelmi, A. E.: *ibid.*, p. 69.

CARBOHYDRATE METABOLISM IN PREGNANCY: THE DEGRADATION OF INSULIN BY EXTRACTS OF MATERNAL AND FETAL STRUCTURES IN THE PREGNANT RAT[1]

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ABSTRACT

Cell-free extracts have been prepared from livers, placentas, and fetuses of pregnant rats on day 12, 15, 18 and 21 of gestation and from livers of non-pregnant control animals. With the use of I^{131}-insulin and nitrogen reference standards, the insulin-degrading capacities of these structures have been compared on the basis of activity per unit mass (specific activity) and activity per total tissue (total potential activity). It has been demonstrated that mechanisms for the proteolytic degradation of insulin are present within the conceptus throughout gestation. Placental specific activity remained constant and at all times equal to that of maternal and control liver. Thus, for pooled placentas, total potential activity paralleled the increase in placental mass to reach one-third the value of maternal liver at term. Fetal specific activity increased late in pregnancy to approximately twice the level found in the other tissues. In isolated fetal parts, activity was greatest in fetal skin. At term, the total potential activity of the pooled fetuses was almost three times as great as that of maternal liver. The demonstration of insulin-degrading systems in rat placenta and fetus at multiple intervals during pregnancy lends further support to the thesis that proteolysis of hormones within the conceptus may be of significance in the gestational changes in endocrine economy.

THE latter half of human pregnancy is frequently accompanied by a) resistance to exogenous insulin (1); b) precipitation of manifest diabetes mellitus in prediabetic subjects (2–4); and c) enhancement of maintenance requirements for insulin in diabetic patients (2–4). Within the past, these diabetogenic effects of pregnancy (4, 5) have been attributed to an enhanced elaboration of hormonal contra-insulin factors. In another communication, the authors have reviewed the clinical observations and have proposed that the phenomena could also result, at least in part, from intrauterine mechanisms for the proteolysis of protein hormones (6). In

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the presence of the conceptus as an *added* site for the irreversible inactivation of protein hormones, the maintenance of equilibrium in pregnancy would necessitate an increase in the synthesis of these principles unless extrauterine disposition or requirements were reduced commensurately. The first experimental support for this postulate was derived from the recent demonstration of an insulin-degrading system in human term placenta (7). In the United States, detailed documentation of placental and fetal activities throughout human gestation is obviously impossible. Therefore, the present studies were initiated to see if such information could be obtained with conventional laboratory animals.

The experiments have indicated that proteolytic inactivation of insulin can be effected by rat placenta throughout gestation and that similar enzymatic activity is also present in the rat fetus. Per unit nitrogen, the insulin-degrading capacity of the rat placenta equals that of the maternal liver but is not as great as that of fetal structures. A portion of the data has previously been presented in abstract form (8).

MATERIALS AND METHODS

1. *Animals:* Pregnant rats (Sprague-Dawley descendants) and non-mated littermate females to serve as controls were obtained from the Charles River Breeding Laboratories (Brookline, Massachusetts). The mating of 200 to 250 gm. rats had been instituted during a 12 hour interval and gestation was dated from the time of sexual exposure. Ten days following mating, pregnant and control animals were received in the Thorndike Memorial Laboratory and maintained in individual cages with free access to Purina pellets and water until sacrifice. Groups of 4 to 6 pregnant and 3 non-pregnant control rats were sacrificed on the 12th, 15th, 18th and 21st day of gestation by a blow on the head, decapitation and exsanguination.

2. *Preparation of Tissue Extracts:* Immediately following sacrifice, the entire liver was removed and an aliquot was frozen on powdered, solid CO_2. Thereafter, the uterus was excised, opened and indivudal fetuses and placentas were dissected free. Representative placentas and intact fetuses were frozen. The remainder and the rest of the liver were weighed together with the frozen aliquots in order to obtain estimates of the total mass of liver, placentas and fetuses. A Sartorius automatic balance with an accuracy of ± 0.1 mg. was employed. 21-day old fetuses were subdivided into liver, abdomen (after removal of liver), head, thorax and limbs for separate analysis of component fetal parts. In addition, portions of skin from 21-day old fetuses and livers from one-day old neonatal rats were frozen for assay.

The frozen weighed tissue aliquots were homogenized with 5 volumes of 0.1 M phosphate buffer (pH 7.4) in ground-glass Potter-Elvehjem homogenizers. Homogenates were centrifuged for 30 minutes at 15,000×g., 4° C and the soluble supernatant was employed (i.e., "15,000×g. tissue extract"). Thus, each ml. of extract represented the equivalent of 166 mg. of initial wet tissue weight. Experiments were conducted immediately following the preparation of the "15,000×g. extracts." In separate studies designed to assess the stability of these preparations, portions of extracts were a) boiled for 10 minutes, or b) stored at −18° C for 14 days prior to assay. A micro-Kjeldahl technique was employed to measure the nitrogen content of each "15,000×g. tissue extract."

3. *Incubation Procedure:* Following temperature equilibration, one ml. of the "15,000 ×g. extract" was combined with one ml. of 0.1 M pH 7.4 phosphate buffer to yield a

2 ml. volume reaction mixture. The phosphate buffer had been supplemented previously with human serum albumin (HSA), crystalline zinc insulin,[3] and tracer quantities of I^{131}-labeled insulin[4] to yield 6.25 mg. HSA and either 25 or 100 μg. insulin per ml. of final reaction mixture. The HSA was designed to minimize adsorption to glassware and spontaneous degradation of insulin as well as to provide adequate amounts of carrier protein for precipitation (v.i.). Details of preparation of insulin-HSA mixtures have been outlined elsewhere (6). Incubation was performed at 38° C in open 20 ml. beakers shaken at a rate of 100 cycles/minute in a Dubnoff metabolic incubator.

4. *Assay of Insulin Degradation:* Aliquots of reaction mixture were removed at timed intervals and introduced into 13×100 mm. tubes containing chilled 10% trichloracetic acid (TCA). Following centrifugation, the supernatant was quantitatively decanted into 13×100 mm. tubes and the sediment was resuspended with 10% TCA for washing. Supernatants were pooled and the washed sediment was dissolved in an equivalent volume of 30% KOH. The TCA-soluble and TCA-insoluble radioactivities were counted in a well-type scintillation detector with a sensitivity of 1×10^6 cpm/μc. I^{131}. Control vessels were incubated concurrently without tissue extract and aliquots of media were fractionated into TCA-soluble and TCA-insoluble moieties. After appropriate correction for the control vessels, insulin degradation by tissue extracts was assessed on the basis of the evolution of non-precipitable I^{131}

$$\text{(i.e. \% insulin degradation} = \frac{\text{TCA-soluble radioactivity}}{\text{TCA-soluble} + \text{TCA insoluble radioactivity}} \times 100\text{)}.$$

In some experiments, ascending paper chromatography in butanol:acetic acid:water (78:10:12) was employed for further characterization of reaction products, Portions of unfractionated reaction mixture were directly applied to Whatman #1 filter paper after 30 minutes of incubation, dried, and supplemented with carrier quantities of L-monoiodotyrosine (MIT), L-diiodotyrosine (DIT), L-thyroxine (T_4), L-triiodothyronine (T_3), and ionic iodide. Marker compounds were localized by spraying with diazotized sulfanilic acid and palladium chloride following chromatography, and the radioactive products of the degradation of I^{131}-insulin were localized by radioautography and strip-scanning as described previously (6).

5. *Comparison of Insulin-Degrading Capacities of Different Tissues*: A standardized incubation of 30 minutes and a standardized insulin concentration in the reaction mixture of 100 μg. insulin/ml. was employed to compare activities of different structures. Relative activities were contrasted in two ways: Insulin degradation per unit mass of tissue was expressed on the basis of "specific activity" (S.A.) wherein

$$\text{S.A.} = \frac{\text{Non-Precipitable } I^{131} \text{ (\% of total } I^{131})}{\text{mg. N in 1.0 ml. "15,000} \times \text{g. extract"}}.$$

Potential insulin degradation per total mass of tissue was expressed on the basis of "total potential activity" (T.P.A.) wherein:

T.P.A. = S.A. × mg. N which would have been obtained if the entire tissue had been employed to prepare the "15,000 × g. extract." In the case of placenta and fetus, the pooled weights of all of the placentas and fetuses from a single animal were employed to calculate T.P.A.

The choice of the 100 μg./ml. concentration of insulin and the 30 minute incubation period for standardized comparison of different tissues was based on the fact that insulin

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[3] Crystalline zinc insulin (lot #499667) assaying 25 units/mg. was generously provided by Dr. O. K. Behrens and Dr. C. W. Pettinga of Eli Lilly Company.

[4] I^{131}-labeled crystalline beef insulin was purchased from Abbott Laboratories, Oak Ridge, Tennessee.

TABLE 1. INSULIN DEGRADATION BY TISSUES OF PREGNANT RATS: EFFECTS OF INSULIN CONCENTRATION

Reaction Mixture: 2 ml pH 7.4 phosphate buffer containing "15,000×g. extracts" of maternal liver, placenta or fetus; constant tracer quantities of I^{131}-insulin, and crystalline unlabeled insulin to a final concentration of 25 or 100 μg./ml. Aliquots of reaction mixture were analyzed following 5 and 30 minutes of incubation at 38° C.

		Non-precipitable I^{131} (% of total I^{131})			
Insulin (μg./ml.)		25		100	
Incubation (mins.)		5	30	5	30
Extract	No. of animals				
Maternal Liver	12	30.7 ± 5.9*	66.1 ± 4.5	11.7 ± 4.0	35.2 ± 5.6
Placenta	6	15.9 ± 4.0	42.4 ± 3.9	7.4 ± 2.4	19.0 ± 2.9
Total Fetus	4	17.9 ± 3.6	55.8 ± 11.0	7.8 ± 2.6	22.9 ± 4.5

* Mean ± standard deviation.

degradation by all of the tissues under these conditions was a) well below the asymptotic values obtained at lower insulin concentrations, and hence closer to linear reaction velocity and yet b) sufficiently great to insure against significant co-precipitation error during processing with TCA. Since insulin-degrading activity is principally localized in the non-particulate soluble cytoplasm (6, 9–12), the use of the nitrogen content of the "15,000 × g. extracts" to estimate S.A. and T.P.A. appears justified. However, it is appreciated that the nitrogen values of the various "15,000 × g. extracts" could be varyingly diluted with inert components derived from occluded plasma proteins. The preliminary exsanguination of donor animals was instituted to minimize this possible contribution of differences in tissue vascularity.

RESULTS

1. *Insulin Degradation by the Conceptus:* Placental and fetal extracts, like extracts of maternal liver, effected an evolution of TCA-soluble radioactivity during incubation with I^{131}-insulin (Table 1). Insulin degradation was conditioned by the duration of incubation and inversely proportional to the total amount of insulin (i.e. labeled and unlabeled) in the reaction mixture (Table 1). The chromatographic mobilities of the radioactive reaction products mostly coincided with the migrations of the iodinated tyrosine markers (Fig. 1). Only a small amount of ionic iodide was observed during chromatography of the incubated reaction mixtures thus validating that the formation of TCA-soluble radioactivity from I^{131}-insulin by the conceptus parallels proteolysis of the insulin molecule.

The enzymatic properties of the placental extracts were examined further. At fixed concentrations of insulin, the degradation of insulin was proportional to the quantity of placental extract added to the reaction mixture (Table 2). Boiling reduced the activity of placental extracts as did storage for 14 days at -18° C (Table 2). A small proportion of the placental activity was shown to be heat-stable (Table 2).

2. *Comparison of the Insulin-Degrading Capacities of Different Tissues at Various Times in Gestation:*

A. *Specific Activity:* Mean values for day 12, 15, 18 and 21 of gestation are illustrated in Figure 2. S.A. of maternal liver remained essentially constant throughout the period of observation and was not significantly different from the values for the livers of control, non-pregnant rats. The day of gestation listed below each set of control values in Figure 2 refers to the experimental group with which these control analyses were obtained. The relative constancy of the control data secured on four separate occasions affords an index of the reproducibility of the assay procedure.

The placenta at 12 days is a cone-like structure which cannot be separated from the embryonic investments in which it is developing. The em-

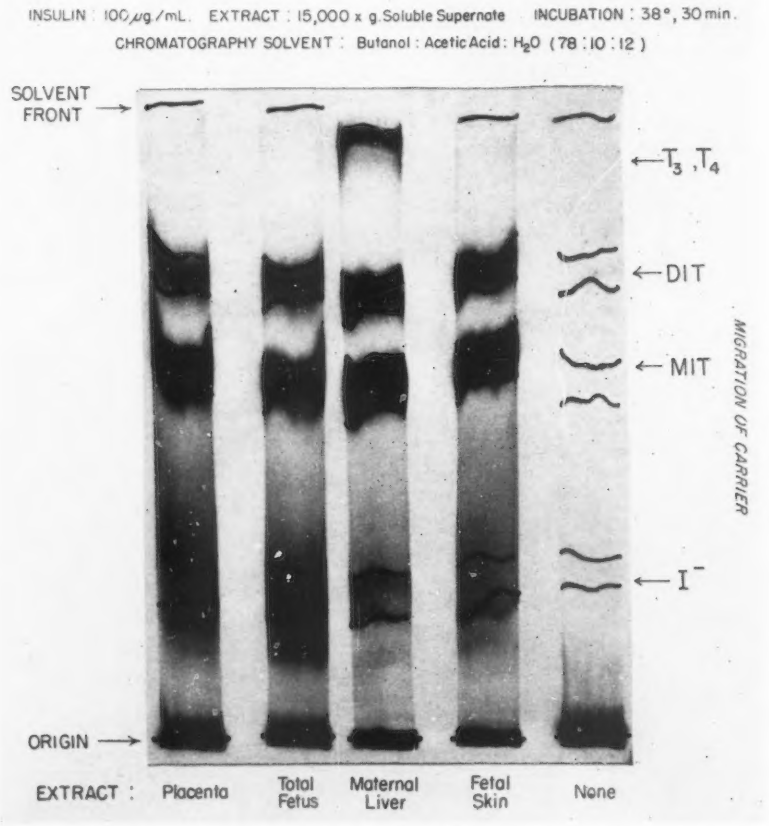

FIG. 1. Chromatographic Characterization of the Products of Insulin Degradation by Extracts of Maternal and Fetal Structures: Aliquots of reaction mixture were chromatographed in butanol; acetic acid: water as outlined in text following 30 minutes of incubation. The heavy black lines denote the position of marker compounds localized by spraying with diazotized sulfanilic acid and palladium chloride. Radioactivity at origin represents chiefly undegraded I^{131}-insulin.

TABLE 2. INSULIN DEGRADATION BY TISSUES OF PREGNANT RATS: EFFECTS OF BOILING AND STORAGE ON ACTIVITY OF PLACENTAL EXTRACTS

Increasing quantities of extract were added to a trace amount of I^{131}-insulin supplemented with crystalline insulin to a concentration of 25 μg./ml. in a final volume of 2.0 ml. Incubation was carried out at 38° C for 10 minutes. Fresh "15,000×g. placental extract" was a) incubated immediately (unboiled), b) boiled for 10 minutes prior to incubation (boiled) and c) stored at −18° C for 14 days prior to incubation (stored).

Extract	Ml. added	Non-precipitable I^{131} (% of total I^{131})
Unboiled	0.25	9.4
	0.50	16.7
	1.00	27.1
Boiled	0.25	2.3
	0.50	4.0
	1.00	3.9
Stored	0.25	2.4
	0.50	10.9
	1.00	18.5

bryo at this stage is about 5 mm. long. At 12 days, therefore, the embryo together with the membranes and the primitive placental cone were homogenized as a whole to obtain "fetal" extracts, while the uterine decidua into which this structure is implanted was homogenized to prepare the "placental" extracts. (After the 12th day, the placenta and fetus were

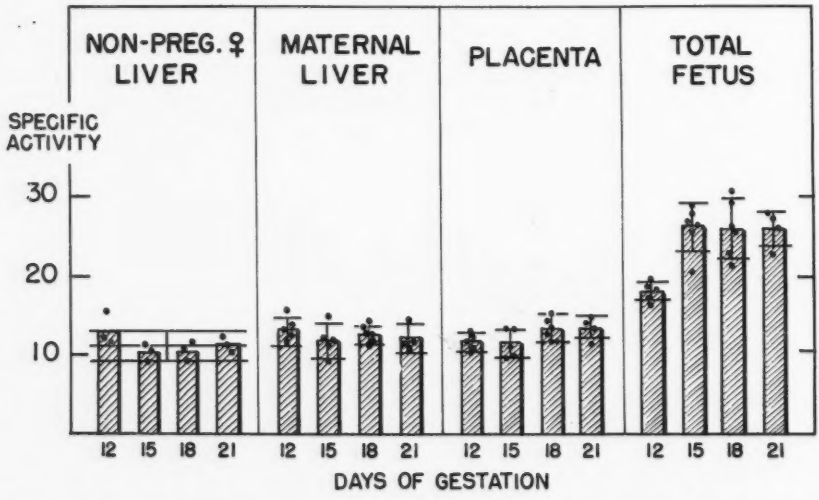

FIG. 2. Specific Activities for Insulin Degradation by Extracts of Maternal Liver, Placenta and Fetus, and Liver of Non-pregnant Control Rats: At each interval in gestation, a group of non-pregnant control animals was assayed concurrently. Mean values are depicted by heights of the bar graphs; the bracketed lines denote ± one standard deviation. The observations obtained at each of the four intervals were pooled to obtain the mean ± one standard deviation values for control liver which are indicated by the horizontal lines.

easily dissociated for separate analysis.) The S.A. of placental extracts on day 12, 15, 18 and 21 of gestation was equal to that of maternal liver while fetal S.A. exceeded hepatic values at 12 days and was twice as great as that of liver (and placenta) at later points in development (Fig. 2).

The inordinately high S.A. of extracts of total fetus prompted more detailed analyses of isolated parts of 21-day old fetuses. Fetal liver, although more active than maternal liver, exhibited less activity than the total fetus (Fig. 3). The S.A. of "15,000 × g. extracts" of other structures such as head, thorax, abdomen (after removal of the liver), and limbs were equal or greater than the values for the total fetus. This finding suggested that greater activity must be present in some tissue common to these various parts. Accordingly the fetal skin was tested and was found to have the

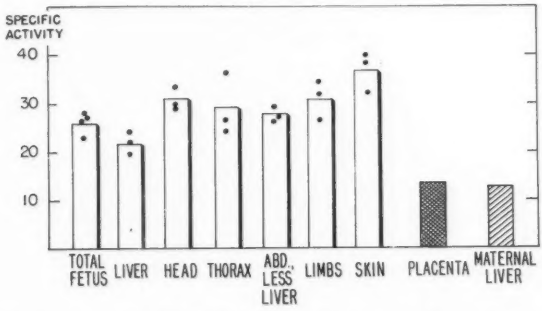

FIG. 3. Specific Activities of Individual Fetal Structures: Extracts were prepared with tissues obtained on day 21 of gestation. Values for 21 day maternal liver and placentas are included for comparative purposes.

highest activity of all tissues examined (Fig. 3). The average S.A. values for 21-day placenta and maternal liver are presented in Figure 3 to emphasize the disparately great insulin-degrading activity of fetal extracts. The presence of much proteolytic activity in adult rat skin has been described previously (13).

Livers of six new-born rats were also examined for insulin-degrading capacity. S.A. of neonatal liver averaged 18.4 ± 1.7,[5] a value midway between that of fetal and maternal liver.

B. *Total Potential Activity:* As defined in the Methods section, T.P.A. at any given level of S.A. will depend upon a) the total weight of tissue, and b) the mg. N in a "15,000 × g. extract" prepared from a weighed aliquot of that tissue.

From day 12 to 21 of gestation, maternal liver weight remained relatively constant and averaged 11.79 ± 2.03 gm.[5] (Table 3). The livers of control animals weighed significantly less and averaged 9.03 ± 0.82 gm.[5]

[5] Mean ± Standard Deviation.

TABLE 3. AVERAGE VALUES FOR THE NITROGEN CONCENTRATION OF "15,000×G. EXTRACTS" AND THE TOTAL WET WEIGHTS OF MATERNAL LIVER, POOLED PLACENTAS* AND POOLED FETUSES* AT VARIOUS TIMES DURING GESTATION IN THE RAT

Day	No. of rats	Litter size (range)	Maternal liver		Placentas		Fetuses	
			Total weight (gm.)	Nitrogen (mg./ml.)	Total weight (gm.)	Nitrogen (mg./ml.)	Total weight (gm.)	Nitrogen (mg./ml.)
12†	5	10 (8–12)	11.36	3.52	0.891†	1.69	0.791†	1.73
15	6	11 (7–16)	11.64	3.49	2.27	2.11	2.12	0.88
18	6	11 (7–13)	12.32	3.58	4.28	2.10	9.67	1.19
21	4	9 (7–12)	10.98	3.71	4.90	2.27	37.8	1.39

* Values for total wet weights of pooled placentas and fetuses obtained by multiplying the weight of individual structures by the number of placentas and fetuses in each litter.
† See text for details concerning the isolation of 12 day "placentas" and "fetuses."

($P < 0.01$). Nitrogen concentrations of "15,000 × g. extracts" of maternal and control livers were not significantly different and averaged 3.56 ± 0.26 mg. N/ml.[and 3.63 ± 0.30 mg. N/ml.[5] respectively ($P > 0.5$). Thus, solely by virtue of the weight differential, the T.P.A. of maternal liver significantly exceeded the value for control liver ($P < 0.01$) (Fig. 4).

Derived estimations of T.P.A. for maternal liver, total placentas, and total fetuses on day 12, 15, 18 and 21 of gestation are shown in Figure 4. The data which were employed for these calculations are summarized in Table 3. As can be seen, the placentas gained rapidly in weight from the 12th to 18th day but increased little thereafter, whereas the fetuses continued to grow to term at a progressively increasing rate. The nitrogen concentration of placental extracts was relatively constant throughout placental development whereas the concentration of nitrogen in fetal extracts rose progressively from the 15th to 21st day. Thus, placental T.P.A. paralleled the increase in placental mass to reach a level which approximated one-third that of maternal liver at term. On the other hand, the rate of increase of fetal T.P.A. exceeded the rate of increase of fetal mass. Fetal T.P.A. at term was almost three times as great as that of maternal liver (Fig. 4).

DISCUSSION

The present studies have demonstrated that cell-free extracts of rat placenta and fetus can effect proteolysis of crystalline insulin. Like the extensively characterized "insulinase" systems of rat and beef liver (9–12, 14–17), and human placenta (6, 7), the insulin-degrading activity of rat placenta and fetus diminishes with storage, and is largely, although not entirely, destroyed by heating. Moreover, in rat placenta and fetus, as in the other structures, the heat-labile component conforms to an enzymatic mechanism and cleaves I^{131}-insulin into proteolytic products which approximate the chromatographic mobilities of iodinated tyrosines. Whether similar or even identical enzymes are involved in all of these systems is beyond

the scope of the present studies and should be deferred until purer preparations are available. For the moment, however, the biochemical considerations need not detract from the physiological implications of the available data.

The authors have employed two arbitrary criteria to compare the relative insulin-degrading capacities of intra- and extra-uterine structures during 30 minutes of incubation in systems containing 100 μg. insulin/ml. *In vitro* activity per unit mass of tissue has been designated as "specific activity" (S.A.)—i.e. insulin degradation per mg. N in a 15,000 × g. extract of a weighed tissue aliquot. The maximum activity which a given structure could potentially exhibit *in vivo* has been called "total potential activity" (T.P.A.). Herein, allowance has been made for total tissue mass by multiplying S.A. by the mg. N which would have been obtained if the entire tissue had been employed to prepare the 15,000 × g. extract. On the basis of S.A., placental insulin-degrading capacity remained constant throughout

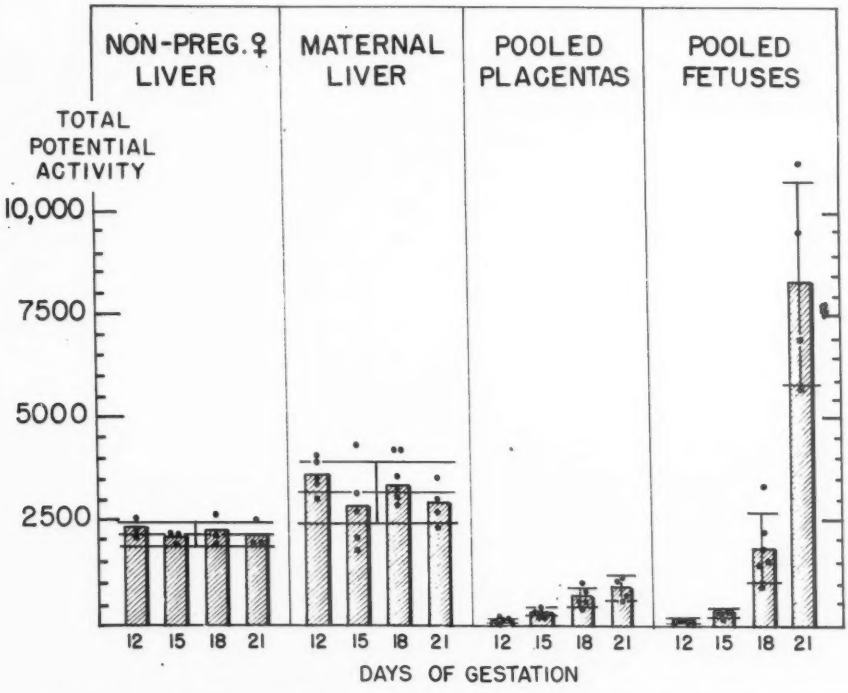

FIG. 4. Total Potential Activities of Maternal and Fetal Structures During Gestation in the Rat: Mean values are depicted by heights of the bar graphs; bracketed lines denote ± one standard deviation. The T.P.A. levels obtained for maternal and control livers at each of the four intervals were pooled to derive the mean ± one standard deviation values for these tissues which are indicated by the horizontal lines.

gestation and equaled that of liver. Fetal S.A. at all times exceeded the liver values. The insulinolytic capacity of the conceptus can best be appreciated when it is recalled that "insulinase" is more abundantly concentrated in the liver of the non-pregnant rat than in any other extrauterine structure (9).

The calculation of T.P.A. facilitated correction for the gestational changes in the mass of intrauterine structures. Thus, placental T.P.A. rose from negligible levels to about one-third the value of maternal liver at term. Similarly, although the S.A. of maternal and control livers were equal, the T.P.A. of the former exceeded that of the latter, because of the increase in the mass of maternal liver during pregnancy. From this it may be inferred that the development of intrauterine mechanisms for insulin degradation is not accompanied by a commensurate reduction in the extrauterine potential. Gestational changes were more pronounced in fetal tissues than in any other structure. Herein, both S.A. and mass increased during pregnancy so that T.P.A. at term for pooled fetuses was almost three times as great as that of maternal liver.

Precise physiological interpretation of the *in vitro* findings must be done with caution. Although T.P.A. represents a maximum value for insulin-degrading capacity, it need not correspond to the *true* activity *in vivo*. In the intact animal, the absolute availability of substrate, such as insulin, for intracellular enzymatic processes, such as proteolysis, will be conditioned by regional blood flow, transcapillary exchange and transcellular penetration. For the rat conceptus, none of these phenomena have been adequately documented. However, assuming that perfusion and permeability per unit mass of placenta remain constant throughout gestation, then the present data would suggest that absolute placental mass is the chief determinant of the placental contribution to maternal insulin economy. The effects upon maternal insulin would be negligible early in pregnancy and would become increasingly significant as placental development progressed. On the other hand, for the fetus, developing in parallel with the placenta, placental insulin degradation would be of importance throughout gestation because of the strategic interposition of the placenta between maternal and fetal circulations.

The demonstration of great insulinolytic activity in fetal extracts exemplifies the dangers inherent in attempts to extrapolate *in vitro* observations to events *in vivo*. For the rat, this fetal activity does not seem to play a major role in the disposition of maternal insulin since the authors have been unable to demonstrate appreciable transplacental passage of insulin in the intact animal (8). This does not preclude the possibility that fetal proteolysis of insulin might be of consequence to maternal insulin economy in species in which insulin can cross the placenta freely. The precise physiological significance of the inordinately great insulin-degrading capacity of the fetus is obscure. Whether it is related to the active turnover of proteins

in fetal structures, whether it obtains in other species besides the rat, and finally, whether it may be implicated in the reported fetal resistance to exogenous insulin (18, 19) must be deferred for further inquiry.

For the moment, however, the specific demonstration of mechanisms for insulin degradation in the rat placenta and fetus throughout gestation lends further support to the more general thesis that proteolysis within the conceptus warrants consideration in the endocrine interactions of pregnancy.

REFERENCES

1. Burt, R. L.: *Obst. & Gynec.* **7:** 658. 1956.
2. Pedersen, J.: *Acta endocrinol.* **9:** 342. 1952.
3. Bergqvist, N.: *Acta endocrinol.* **15:** 166. 1954.
4. Hagbard, L.: *Acta obst. & gynec. Scandinav.* **35:** Suppl. I. 1956.
5. Hoet, J. P.: *Diabetes* **3:** 1. 1954.
6. Freinkel, N. and C. J. Goodner: *J. Clin. Invest.* in press.
7. Freinkel, N. and C. J. Goodner: *J. Clin. Invest.* **37:** 895. 1958.
8. Goodner, C. J. and N. Freinkel: *Clin. Res.* **7:** 247. 1959.
9. Mirsky, I. A.: In *Ciba Foundation Colloquia on Endocrinology.* Boston, Little, Brown and Company, 1953, vol. 6, p. 263.
10. Vaughan, M.: *Biochim. et Biophys. Acta* **15:** 432. 1954.
11. Beyer, R. E.: *Acta endocrinol.* **19:** 309. 1955.
12. Narahara, H. T., H. H. Tomizawa, R. Miller and R. H. Williams: *J. Biol. Chem.* **217:** 675. 1955.
13. Martin, C. J. and A. E. Axelrod: *J. Biol. Chem.* **224:** 309. 1957.
14. Mirsky, I. A., G. Perisutti and F. J. Dixon: *J. Biol. Chem.* **214:** 397. 1955.
15. Tomizawa, H. H., M. L. Nutley, H T.. Narahara and R. H. Williams: *J. Biol. Chem.* **214:** 285. 1955.
16. Tomizawa, H. H. and R. H. Williams: *J. Biol. Chem.* **217:** 685. 1955.
17. Mirsky, I. A. and G. Perisutti: *J. Biol. Chem.* **228:** 77. 1957.
18. Schlossmann, H.: *J. Physiol.* **92:** 219. 1938.
19. Passmore, R. and H. Schlossmann: *J. Physiol.* **92:** 459. 1938.

NOTES AND COMMENTS

ALTERATION OF PITUITARY ACTH SECRETION IN RATS AFTER INJECTION AND HANDLING[1]

ABSTRACT

Pituitary ACTH concentrations were measured by an *in vitro* steroidogenic method in rats at intervals following a scald stress. Depletions of 52 and 66% of control ACTH levels were observed 1 and 4 hours after stress in intact animals. Other rats pre-treated with saline injections for 7 days prior to stress showed ACTH depletions of 18 and 25% of control levels 1 and 4 hours after stress. An altered pattern of ACTH depletion was noted also in adrenalectomized rats injected prior to stress. Saline injections had no measurable effect on resting pituitary ACTH concentration, adrenal weight or adrenal responsiveness to exogenous ACTH. Treatment with injections prior to stress may increase the capacity of the pituitary gland to synthesize ACTH rapidly in response to stress.

During the course of recent experiments concerned with factors regulating the secretion of pituitary adrenocorticotropin (ACTH) (1–3), it became necessary to determine the effect of injecting and handling of the animals, as distinguished from that induced by the injected substance itself, on ACTH synthesis and release in intact and adrenalectomized rats.

METHODS

Male Sherman rats, 100–140 gm., were maintained on a diet consisting of Purina laboratory chow and water *ad libitum* in a constant temperature room for at least one week prior to use. Anterior pituitary glands from each treatment group in Experiments 1 and 2 were pooled and extracted by the method of Birmingham *et al.* (4), and subsequently assayed for ACTH concentration by an *in vitro* steroidogenic technique described previously (1). The absolute potencies of the pituitary extracts of the appropriate unstressed control animals are expressed as concentrations of ACTH per mg. anterior pituitary tissue, and are presented in Table 1. These extracts were then employed as standards in each experiment and assigned arbitrary potencies of 100%. The pituitary ACTH concentrations in the various experimental groups are expressed as per cents of their respective controls. These percentages represent an arithmetic conversion of the mean log potency ratios obtained. The absolute potencies of the various experimental groups can be derived by simple multiplication of these per cents by the absolute potencies of the control extracts. The statistical significance of differences, when determined from per cent changes, is unaltered when calculated from changes in absolute potencies. Comparisons of differences in ACTH potency were determined from the mean log potency ratios and their respective log standard errors ($\overline{M} \pm s_{\overline{m}}$) and not upon the arithmetic percentages (antilog $\overline{M} \pm 2.303\ s_{\overline{m}}$ antilog $\overline{M}$) presented in the tables. Statistical analyses were performed according to the methods of Snedecor (5) and Bliss (6).

Experiment 1A: Rats which had not been handled or treated prior to the day of the experiment were subjected to a stress, which consisted of immersion of the anesthetized rat to the neck in 70° C water for 5 seconds. Thus, the stress consisted of anesthesia plus scald. Groups of these animals were sacrificed at 1, 2, 4, 8 and 24 hours following stress. Animals which had not been stressed served as controls. Another group of rats was injected daily with 0.2 ml. physiological saline subcutaneously for 7 days and the scald stress was applied 24 hours after the last injection. Controls for this group consisted of animals which had been similarly injected but not scalded. The pituitary ACTH concentrations were determined at 1 and 4 hours after stress (Table 2).

Experiment 1B: Under conditions identical to those in Experiment 1A, the pituitary ACTH concentrations of injected and uninjected groups of rats sacrificed 4 hours after the scald stress were compared to ACTH concentrations of respective unstressed control groups (Table 2).

Experiment 2: A group of animals was adrenalectomized and maintained on 1% saline drinking water. Eight days following adrenalectomy, the scald stress was applied and pituitary ACTH concentrations measured 1 and 4 hours following stress (Table 3). The control animals consisted of unstressed rats, adrenalectomized at the same time as the stressed rats. Another group of adrenalectomized rats received subcutaneous injections of 0.2 ml. physio-

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TABLE 1. ABSOLUTE ACTH POTENCIES OF CONTROL PITUITARY EXTRACTS USED IN EXPERIMENTS 1 AND 2

Expt.	Uninjected group	Injected group
1A	85*	80
1B	77	72
2	144	142

* mμ ACTH per mg. anterior pituitary tissue.

logical saline for 7 days following adrenalectomy and the scald stress was applied 24 hours following the last injection. Pituitary ACTH concentrations were measured 1 and 4 hours following stress (Table 3). The control animals, adrenalectomized at the same time, had also been injected but not stressed.

RESULTS

The pituitary ACTH concentrations of the various unstressed control groups were compared in a separate assay, and absolute potencies were determined (Table 1). ACTH levels in the intact injected and uninjected groups did not differ, nor did the ACTH concentrations in the adrenalectomized injected and uninjected groups differ, in each instance, by more than 6%, a statistically insignificant difference. Previous handling and injecting does not in itself produce a measurable change in pituitary ACTH concentration. Adrenalectomy produced the expected rise in pituitary ACTH concentration, with the adrenalectomized rats having a potency of 1.7 times that of the unoperated animals.

TABLE 2. EFFECTS OF REPEATED INJECTIONS OF SALINE ON PITUITARY ACTH CONCENTRATION AFTER SCALD STRESS IN INTACT RATS

	Pituitary ACTH concentration as % unstressed level[1,2]			
	Experiment 1A		Experiment 1B	
Time after stress (hours)	Uninjected	Injected	Uninjected	Injected
Unstressed	100% (4)	100% (4)	100% (4)	100% (4)
1	48 ± 9[3] (4)	82 ± 9[4] (3)		
2	44 ± 7[3] (6)			
4	34 ± 7[3] (4)	75 ± 12[4] (4)	32 ± 5[3] (3)	70 ± 9[3] (4)
8	42 ± 8[3] (4)			
24	38 ± 7[3] (3)			

[1] Antilog $\overline{M}$ + 2.303 $s_{\overline{M}}$ Antilog $\overline{M}$.
[2] Numbers of animals studied indicated in parentheses.
[3] $P \leqq .01$.
[4] $P \leqq .05$.

TABLE 3. EFFECTS OF REPEATED INJECTIONS OF SALINE ON PITUITARY ACTH CONCENTRATION AFTER SCALD STRESS IN ADRENALECTOMIZED RATS

	Pituitary ACTH concentration as % unstressed level[1,2]	
Time after stress (hours)	Uninjected	Injected
Unstressed	100% (3)	100% (4)
1	67 ± 7[3] (3)	97 ± 14 (4)
4	69 ± 6[3] (3)	67 ± 9[3,4] (3)

[1] Antilog $\overline{M}$ ± 2.303 $s_{\overline{M}}$ Antilog $\overline{M}$.
[2] Number of animals studied indicated in parentheses.
[3] $P \leqq .01$.
[4] This value has been presented in a previous publication (3).

Application of a scald stress, in the intact uninjected rat, results in a depletion in ACTH concentration to levels less than half of those measured in unstressed controls (Table 2). This reduction is present 1 hour following application of the stress; it persists for at least 24 hours, reflecting the prolonged trauma inflicted by the severe scalding procedure. On the other hand, animals pre-treated with saline injections exhibited a modified pattern of ACTH depletion after stress. Although reductions in ACTH concentration were observed at 1 and 4 hours after scalding, these reductions were significantly less ($P \leqq .01$) than those observed at equivalent intervals in animals which had not been injected prior to stress. The significance of these differences is unaltered even when appropriate corrections are made for minor differences in pre-stress ACTH concentrations between the two groups.

Both groups of adrenalectomized rats, pre-treated with saline injections or untreated, demonstrated pituitary ACTH depletions of similar degree 4 hours after stress, when each was compared to its own unstressed control group (Table 3). However, at 1 hour following stress, no change in pituitary ACTH concentration was observed in the injected group, whereas the uninjected animals demonstrated significant depletion.

Adrenal responsiveness to ACTH was not changed by the saline injections, since animals so injected for 7 days, hypophysectomized and tested 24 hours later with 4 m.u. of a standard ACTH preparation given i.v., showed an adrenal ascorbic acid depletion of 160 ± 18 mg./100 gm. adrenal weight; this degree of depletion is very similar to that seen after injection of 4 m.u. of ACTH in hypophysectomized animals not previously injected (7).

Adrenal weight was not significantly increased by 7 days of saline injections. It is unlikely, therefore, that an appreciable increase in the release of pituitary ACTH occurs as a consequence of such treatment.

DISCUSSION

Normal rats apparently react to the severe stress of scalding with a rapid release of preformed ACTH from pituitary stores of the hormone. This is followed by a much longer period during which pituitary ACTH levels remain relatively constant, indicating that synthesis and release of ACTH are in balance. Animals which have been handled and injected with saline prior to stress have considerably less depletion of pituitary ACTH during the 4 hour period following the stress. These animals either release less or synthesize more ACTH in response to stress, compared to uninjected controls. Preliminary data (unpublished) indicates that an ether stress produces equivalent falls in adrenal ascorbic acid in normal and saline-injected animals, implying the release of similar amounts of ACTH in the two preparations.

It is likely that these changes in the pituitary gland's response to stress represent the effects both of the daily saline injection and also the animal handling incident to such injection. The present study does not permit conclusions concerning the relative importance of these two factors or the adequacy of handled but uninjected animals as a control group in an experiment which involves injections.

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REFERENCES

1. KITAY, J. I., D. A. HOLUB AND J. W. JAILER: *Proc. Soc. Exper. Biol. and Med.* **97:** 165. 1958.
2. HOLUB, D. A., J. I. KITAY AND J. W. JAILER: *J. Clin. Invest.* **38:** 291. 1959.
3. KITAY, J. I., D. A. HOLUB AND J. W. JAILER: *Endocrinology* **64:** 475. 1959.
4. BIRMINGHAM, M. K., E. KURENTS, G. J. ROCHEFORT, M. SAFFRAN AND A. V. SCHALLY: *Endocrinology* **59:** 677. 1956.
5. SNEDECOR, G. W.: Statistical Methods. The Iowa State College Press. Ames. 1956.
6. BLISS, C. I.: The Statistics of Bioassay. Academic Press. New York. 1952.
7. SAYERS, M. A., G. SAYERS AND L. A. WOODBURY: *Endocrinology* **42:** 379. 1948.

FATE OF PROGESTERONE INJECTED SUBCUTANEOUSLY IN MICE[1]

ABSTRACT

Ovariectomized adult mice were injected subcutaneously with 1.5 ml. of progesterone in oil at a concentration of 1000 μg./ml. The mice were killed at intervals by rapid exsanguination, and the plasma and the recovered oil were separately bioassayed for progestin. Plasma levels were rather low. Concentrations of progestin in the oil dropped from the initial 1000 Progesterone Equivalents/ml. to 101.2 P.E./ml. at 2¾ minutes, 28.8 P.E./ml. at 6 minutes, and 16.9 P.E./ml. at 10 minutes.

This experiment was designed to determine the rate of disappearance, previously unreported, of progesterone from an oily solution injected subcutaneously. Progesterone disappears rapidly from its vehicle after intraperitoneal injection, and it appears that absorption from the intramuscular depot is swift and efficient (1, 2).

Plasma levels of "free" progestin were also determined.

An abstract of some of these results has already been published (3).

PROCEDURE

Twenty-eight young adult female mice of the highly inbred CHI strain were bilaterally ovariectomized. Eight days later, each mouse while lightly anesthetized with ether received a subcutaneous injection of 1500 μg. of progesterone dissolved in 1.5 ml. of sesame oil. Injection was made through a fine (#27) hypodermic needle to prevent leakage. Although the muscle layer was not sutured at the time of ovariectomy, autopsy of the animals after injection revealed that the muscle incisions had healed and that there was no communication between the subcutaneous oil depot and the peritoneal cavity.

At intervals of from 2¾ minutes to 8½ hours the mice were exsanguinated by dividing the carotid sheath under ether anesthesia, the moment of the division being accepted as the end point of the experiment. The blood from each mouse was drained directly into a centrifuge tube containing 0.05 ml. of 20% sodium citrate solution as an anticoagulant, and was then centrifuged for five minutes. The plasma was separated and added drop by drop with constant stirring to approximately 10 ml. of acetone at 6° C. The acetone-precipitated plasma protein mixture was stored at 6° C for varying periods of time. Immediately after exsanguination, the subcutaneous depot was tapped, and as much oil as possible was aspirated. Recovery of the oil required from 2 to 4 minutes. This oil was centrifuged for two hours. The plasma was prepared for bio-assay for "free" (i.e., ether-acetone soluble) progestin according to the method of Hooker and Forbes (4). Both the "free" plasma progestin and the recovered oil were separately bio-assayed by this technique (4). The latter detects the equivalent of as little as 0.25 μg. progesterone/ml. test material.

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RESULTS

The initial concentration of progesterone in the subcutaneous sesame oil solution in every case was 1000 P.E./ml. One P.E. (progesterone equivalent) equals the activity of one microgram of progesterone in the Hooker-Forbes bioassay. Concentrations of 101.2, 28.8, and 16.9 P.E./ml. recovered oil were found after $2\frac{3}{4}$ minutes, 6 minutes, and 10 minutes, respectively (Fig. 1). After ten minutes the rate of disappearance was somewhat slower; a concentration of 0.2 P.E./ml. recovered oil was reached by three hours. Less than detectable amounts of progestin were present in all except two subsequent samples, concentrations of 0.5 and 1.0 P.E./ml. recovered oil being found after $5\frac{1}{2}$ and $8\frac{1}{2}$ hours.

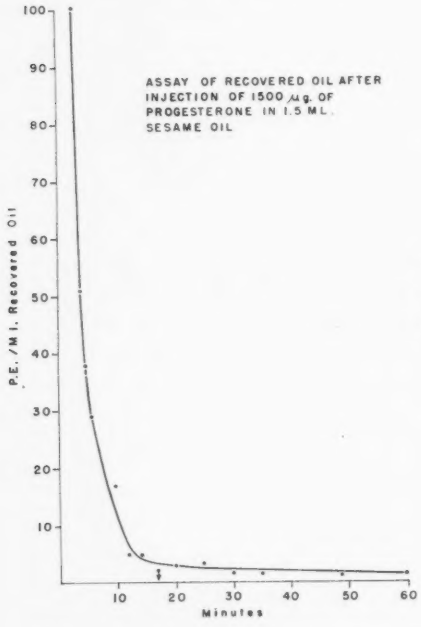

FIG. 1. Each dot represents the sample from one mouse. An arrow on the dot means that less than the indicated amount of hormone was present.

In the mice killed within approximately one hour of injection the depot was small and compact, but as the interval became longer the solution spread into a large area of the subcutaneous tissue. Therefore the surface area of the depot increased as the interval between injection and exsanguination increased.

Plasma concentrations of "free" progestin were quite low during the initial $8\frac{1}{2}$ hours after injection. Levels fluctuated between 0.5 and 1.4 P.E./ml. during the first 6 minutes. Specimens collected between 12 minutes and 3 hours contained less than detectable amounts of progestin.

From $3\frac{1}{2}$ to $8\frac{1}{2}$ hours there were marked fluctuations in the "free" plasma progestin concentrations. At $3\frac{1}{2}$ hours, $4\frac{1}{2}$ hours, and $6\frac{1}{2}$ hours, concentrations of 0.6, 0.4, and 1.4 P.E./ml. plasma, respectively, were found. All other samples obtained after $3\frac{1}{2}$ hours contained less than detectable amounts of progestin.

DISCUSSION

Since the total plasma volume of a young adult mouse is only approximately 1 ml., escape of progesterone into the plasma would not alone seem to be sufficient to explain the rapid disappearance of hormone from the subcutaneous depot.

Hepatic and renal removal and inactivation of part of the progestin which reaches the blood have been demonstrated. An apparently small percentage of the hormone reaching the liver is inactivated by being incorporated into an as yet not definitely characterized molecule. The activity of this fraction can only be measured after release of the progestin by hydrolysis of the plasma protein (5). It is presumed that the mouse, like other animals, may excrete some progesterone and its metabolites in the bile. The kidneys likewise apparently play a part in removing "free" progestin from the blood, as shown by the fact that renal artery-renal vein differences of from 16–94% have been demonstrated in the rabbit and monkey (6). It is presumed that renal inactivation of progesterone may also occur in the mouse.

The distribution of radioactivity following intravenous administration to pregnant women of C^{14}-4-progesterone is highest in the maternal fat tissue (2). Diffusion of progesterone into fat tissue has also been demonstrated by bio-assay (7). It seems reasonable to postulate for the mouse the possibility of direct diffusion from the subcutaneous depot into the surrounding tissue. The fact that in this experiment a slight secondary rise in the plasma levels of "free" progestin was found between 4 and 6½ hours suggests the possibility that there is a slow release of the hormone or its metabolites from the fat tissue. This idea is supported by the fact that after a single intravenous dose of progesterone in human subjects, pregnanediol excretion continues to increase for several days (8).

Statistical analysis of the data concerning the rate of disappearance of hormone from the subcutaneous depot revealed that the rate of decrease was at first rapid and for about the first ten minutes reasonably constant (Fig. 1). However, from then on the rate of decrease diminished slowly until the concentration became low and approximately constant (9). The explanation for this is not immediately evident.

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REFERENCES

1. FORBES, T. R.: *Endocrinology* **64**: 567. 1959.
2. PLOTZ, E. J. AND DAVIS, M. E.: *Proc. Soc. Exper. Biol. and Med.* **95**: 92. 1957.
3. COHEN, S. M.: *Anat. Rec.* **133**: 368. 1959.
4. HOOKER, C. W. AND FORBES, T. R.: *Endocrinology* **41**: 158. 1947.
5. FORBES, T. R. AND HOOKER, C. W.: *Proc. Soc. Exper. Biol. and Med.* **70**: 682. 1949.
6. FORBES, T. R., HOOKER, C. W. AND PFEIFFER, C. A.: *Endocrinology* **47**: 83. 1950.
7. KLEIN, I. AND OBER, K. G.: *Klin. Wchnschr.* **32**: 464. 1954.
8. SOMMERVILLE, I. F. AND MARRIAN, G. F.: *Biochem. J.* **46**: 285. 1950.
9. WHITE, C.: Personal communication.

THE POSTNATAL DEGENERATION OF THE METRIAL (MYOMETRIAL) GLANDS IN THE WHITE RAT

ABSTRACT

The metrial glands of the white rat were measured during the postnatal period. The curve showing the decrease in the size of the glands indicates that degeneration is rapid during the first few days and slower thereafter.

The metrial gland develops during pregnancy in a number of rodents (1). In the rat and mouse it forms a clearly marked, yellow-colored body situated at the uterine at-

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tachment of the mesometrium in the centre of the sub-placental region. The histological and histochemical aspects of its development during pregnancy and its post-partum degeneration in the rat (1–11) and in the mouse (4, 12–13) have been described. The site of the metrial gland is marked for long afterwards—even up to twelve months (15)—by a spot of dark brown pigment. These spots of pigment are frequently referred to as "placentral scars" (6, 8).

Quantitative data on the size of the metrial gland during and after pregnancy seem to be lacking. As a preliminary to experimental studies on the factors concerned in the maintenance and degeneration of the postnatal metrial gland a quantitative study of the normal changes in the size of the gland during the period following parturition was undertaken.

MATERIALS AND METHODS

The animals used were white rats of a strain inbred in our animal house for a number of years. Virgin rats only were used as it is known that "placental scars" of one pregnancy may remain visible during a subsequent pregnancy and they may be difficult to distinguish from those of the later pregnancy (8). As soon as pregnancy was diagnosed by means of abdominal palpation each animal was isolated; the pregnant rats were examined daily and the day on which offspring were found was counted as Day 1 postpartum. Five rats were examined —after being killed by ether—on each postpartum day up to Day 18, after which day it was

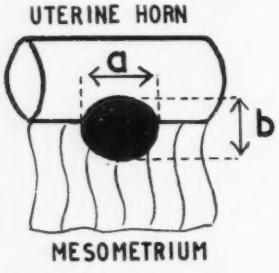

FIG. 1. Schematic representation of a metrial gland showing the diameters measured.

found that the glands were too small for naked eye measurements to be made. In fact, from Day 13 to Day 18 there were many glands whose diameters measured less than 1.0 mm. It was decided to exclude these from the detailed analysis. Rough estimates of the means were made, however, based on the rather inaccurate measurements on these days.

The animals were allowed to suckle their young. In some cases it was found later that the young had "disappeared" from the cage; presumably they had been eaten by the mothers. Such animals were not excluded from the series.

The measurements made consisted of the two diameters of the gland as shown in Figure 1. Diameter "a" runs parallel with the uterine horn and diameter "b" at right angles to the uterine horn. In making these measurements the uterine horn and adjacent mesometrium were allowed to lie on the pulp of the examiner's left index finger, while measurements were made with fine needle-point calipers held in the right hand. Each measurement of each gland was made three times and the mean recorded. The total number of glands measured on each postpartum day is shown in Table 1.

The above measurements were chosen in preference to weighing as it was found that dissecting out the glands was difficult and probably inaccurate.

RESULTS

These are shown in Table 1 and Figure 2.

It will be seen that the curves showing the decrease in the two diameters of the gland are exponential, i.e., the decrease in size is rapid during the first few days and slower thereafter. With regard to Days 13–18, the inaccurate observed estimates were also found to approximate to the expected values.

There is no appreciable difference in the form of the curves for diameters "a" and "b." As far as the relationship between "a"and "b" is concerned, "b" was a little smaller

TABLE I

No. of days post-partum	No. of glands measured	Diameter "a"				Diameter "b"			
		Predicted values mm.	Observed values mm.			Predicted values mm.	Observed values mm.		
			Mean	90% confidence limits			Mean	90% confidence limits	
				Lower	Upper			Lower	Upper
1	41	3.46	3.6	3.3	3.9	3.24	3.3	3.2	3.4
2	45	3.15	3.3	3.1	3.5	2.90	3.0	2.8	3.2
3	45	2.86	2.6	2.3	2.9	2.62	2.5	2.3	2.7
4	41	2.61	2.7	2.4	3.0	2.40	2.4	2.3	2.5
5	29	2.37	2.3	2.1	2.5	2.13	2.3	2.1	2.5
6	53	2.15	2.1	2.0	2.2	1.90	1.8	1.7	1.9
7	36	1.96	2.0	1.8	2.2	1.71	1.7	1.5	1.9
8	48	1.78	1.7	1.6	1.8	1.50	1.4	1.3	1.5
9	42	1.60	1.6	1.5	1.7	1.39	1.2	1.0	1.4
10	42	1.44	1.6	1.5	1.7	1.25	1.2	1.0	1.4
11	37	1.29	1.3	1.2	1.4	1.13	1.2	1.0	1.4
12	34	1.16	1.3	1.2	1.4	1.02	1.1	1.0	1.2
13	—	1.04	—	—	—	0.91	—	—	—
14	—	0.93	—	—	—	0.82	—	—	—
15	—	0.84	—	—	—	0.74	—	—	—
16	—	0.75	—	—	—	0.67	—	—	—
17	—	0.67	—	—	—	0.60	—	—	—
18	—	0.61	—	—	—	0.55	—	—	—

than "a" on all days noted: The correlation coefficient between "a" and "b" was 0.95, indicating a close linear correlation between the two diameters.

Statistical Note

The 90% confidence limits were calculated by using the Student's t distribution. The curve fitting was performed as follows: On the presumption that the curves were

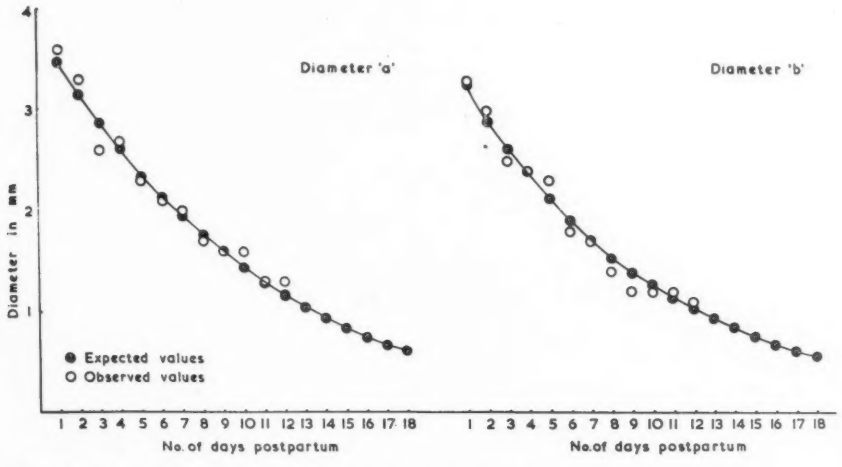
Diameter 'a'
Diameter 'b'
Diameter in mm
4
3
2
1
● Expected values
○ Observed values
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
No. of days postpartum
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
No.of days postpartum

FIG. 2. Measurements of the metrial glands during the postpartum period.

exponential: $d_t = \beta e^{-\alpha t}(i)$, where d = mean of all observations made on each day (t), the parameters α and β were calculated from the logarithmic form of equation (i), namely, $\log d(t) = \log \beta - \alpha t$. The latter equation is that of a straight line and thus the least squares technique was used. It was found that

$$\left.\begin{aligned}\alpha &= 0.09\\ \beta &= 3.8\end{aligned}\right\}\text{in the case of diameter "a"}$$

$$\left.\begin{aligned}\alpha &= 0.12\\ \beta &= 3.6\end{aligned}\right\}\text{in the case of diameter "b"}$$

The conformity between the observed and expected values was examined by the X^2 test and no significant differences were found.

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REFERENCES

1. SELYE, H. AND T. MCKEOWYN: *Proc. Roy. Soc. B.* **119:** 1. 1935.
2. GERARD, P.: *C. R. Soc. de Biol.* **93:** 457. 1925.
3. KISZELY, G.: *Verhandl. d. Anat. Gesell.* **88:** 271. 1939.
4. ASPLUND, J., V. BORRELL AND H. HOLMGREN: *Z. f. mikr. anat. Forsch.* **48:** 478. 1940.
5. BAKER, B. L.: *Proc. Soc. Exper. Biol. Med.* **68:** 492. 1948.
6. CONAWAY, C. H.: *J. Mammalogy* **36:** 516. 1955.
7. WARBRICK, J. G.: *J. Embryol. exp. Morphol.* **3:** 256. 1955.
8. MOMBERG, H. AND C. CONAWAY: *J. Embryol. exp. Morphol.* **4:** 376. 1956.
9. WARBRICK, J. G.: *Q. J. Micr. Sci.* **97:** 11. 1956.
10. WISLOCKI, G. B., L. P. WEISS, M. H. BURGOS AND R. A. ELLIS: *J. Anat.* **91:** 130. 1957.
11. PADYKULA, H. A.: *J. Anat.* **92:** 118. 1958.
12. DENO, R. A.: *Am. J. Anat.* **60:** 433. 1937.
13. LOBO, B. A.: *Contribuicao ao estudo da glandula metrial na camondonga albina.* Rio de Janeiro, 1949.
14. LOBO, B. A.: *Veterinaria* **8:** 22. 1954.
15. DAVIS, D. E. AND J. T. EMLEN: *J. Wildlife Management* **12:** 162. 1948.

EFFECT OF pH AND ROUTE OF ADMINISTRATION ON THE FATTY ACID MOBILIZING ACTIVITY OF GROWTH HORMONE SOLUTIONS[1]

ABSTRACT

Porcine growth hormone prepared by the method of Raben and Westermeyer failed to bring about an increase in plasma non-esterified fatty acid (NEFA) concentration when administered intraperitoneally in acid solution to rats although it was effective in this regard when administered in alkaline solution by the same route. Other growth hormone preparations derived from porcine, bovine and simian pituitaries were equally effective whether injected intraperitoneally in acid or basic solution. When administered intravenously, acid and basic solutions of Raben-Westermeyer hormone brought about comparable increases in plasma NEFA concentrations. It is suggested that the failure of this hormone to mobilize NEFA when administered intraperitoneally in acid solution is due to delayed absorption.

It is now well established that growth hormone, when administered to a variety of species, causes a rapid rise in plasma non-esterified fatty acid (NEFA) concentration (1, 2, 3). This increase in plasma NEFA concentration, in all probability reflects an increase in fatty acid mobilization from adipose tissue (4).

We have recently observed that alkaline solutions of a Raben-Westermeyer porcine growth hormone preparation (5) when injected intraperitoneally into fasting rats will produce the expected elevation of plasma NEFA while acid solutions of the same preparation given at the same dose by the same route will not.

The following studies were undertaken to investigate this problem.

MATERIALS AND METHODS

Female rats (Holtzman) weighing 200 gm. were fasted for twelve hours prior to the administration of 1 mg. of growth hormone. The various growth hormone preparations were dissolved in distilled water at a concentration of 1.0 mg./ml., adjusted to the desired pH with 0.1N HCl or 0.1N NaOH, and were injected intraperitoneally or intravenously under light ether anesthesia. Control animals were injected with 1 ml. of neutral saline by the same route as the experimental animals. Six hours later, after a total fasting period of 18 hours, the animals were anesthetized with ether and blood for NEFA analysis was drawn into a heparinized syringe from the abdominal aorta. NEFA analyses were performed in duplicate by the method of Dole (6) on 1 ml. aliquots of plasma.

One porcine growth hormone used in these studies was prepared by the method of Raben and Westermeyer (5). A second preparation (P205A) was purified from the oxycel filtrate which is the starting material for the Raben-Westermeyer preparation by a new procedure (7). The simian growth hormone was prepared by a slight modification of the procedure described by Wilhelmi (8).

RESULTS

The intraperitoneal administration of porcine (Raben-Westermeyer and P205A), bovine and simian growth hormone in *alkaline* (pH 9.0–9.5) solution induced comparable increases in plasma NEFA concentrations (Table 1). However, the Raben-Westermeyer porcine hormone had no effect on NEFA concentration when administered *intraperitoneally* at a pH of 3 (Fig. 1, Table 1). This apparent loss of activity at pH 3 was not demonstrable in any of the other preparations studied including P205A which is purified from the same starting material as the Raben-Westermeyer hormone (Table 1). The Raben-Westermeyer preparation, however, was equally active in alkaline or acid solutions when it was administered *intravenously* (Fig. 1).

DISCUSSION

Since alkaline (pH 9.0–9.5) and acid (pH 3) solutions of Raben-Westermeyer growth hormone are equally effective in stimulating NEFA mobilization when given intravenously, while only alkaline solutions of this preparation are effective intraperitoneally,

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TABLE 1. THE RESPONSE OF RATS TO VARIOUS GROWTH HORMONE PREPARATIONS ADMINISTERED INTRAPERITONEALLY

Treatment	Administered at pH 9.0–9.5			Administered at pH 3.0–3.5		
	No. of Animals	Plasma NEFA μEq./cc.	P	No. of Animals	Plasma NEFA μEq./cc.	P
Saline	9	0.644 ± .032*		6	0.644 ± .061	
Porcine (Raben-Westermeyer)**	12	0.907 ± .035	<0.01	6	0.629 ± .057	N.S.
Saline	6	0.581 ± .032		6	0.627 ± .026	
Porcine (P205A, Wilhelmi)	6	1.006 ± .056	<0.001	6	0.912 ± .080	<0.01
Saline	6	0.583 ± .029		6	0.627 ± .080	
Bovine	6	0.861 ± .083	<0.01	6	0.927 ± .028	<0.001
Saline	5	0.670 ± .066		5	0.636 ± .063	
Simian	6	1.095 ± .080	<0.001	6	0.995 ± .048	<0.01

* Mean ± S.E.
** All hormone preparations were injected intraperitoneally at a dose of 1 mg./rat.

it can be concluded that the hormone in acid solution may not be absorbed in effective quantities from the peritoneal cavity during the period of the experiment. This behavior could be predicted from a consideration of the solubility characteristics of the Raben-Westermeyer preparation. In concentrations of 1 mg./ml. this hormone is nearly insoluble in the pH range of 3.5 to 9.0. Similar observations have been made by others (9, 10). The other three growth hormone preparations used in this study exhibit a much greater degree of solubility in the region of neutral pH although solutions of these hormones become cloudy when the pH is brought to 5.0–7.5.

The isoelectric points of preparations equivalent to those used in this study have been found to be lower than pH 7 (8, 11). Hence a growth hormone preparation administered in acid solution passes through the region of its lowest solubility in being buffered to physiological pH, thus causing some of the hormone to precipitate out of solution. The amount of hormone remaining in solution and hence available for immediate absorption into the circulation is a function of the solubility of the preparation at physiological pH. In the case of the Raben-Westermeyer hormone, such precipitation

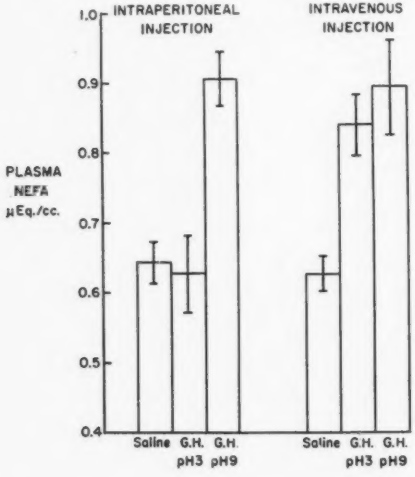

FIG. 1 The NEFA mobilizing effect of 1 mg. of Raben-Westermeyer porcine growth hormone administered intraperitoneally and intravenously in solutions at pH 3 and 9. Each bar represents the mean value obtained from at least 6 rats. The vertical lines indicate the standard errors.

would be extensive and solution and absorption of the material would be delayed. Thus an acute effect of the hormone which depends upon rapid absorption could not occur. Presumably, however, the retarded rate of hormone absorption would be sufficient to stimulate growth over an extended period. While the same considerations would apply to acid solutions of Raben-Westermeyer hormone given intravenously, the much greater dilution of the material in this instance might override its relative insolubility at physiological pH.

Acknowledgments

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REFERENCES

1. Raben, M. S. and C. H. Hollenberg: *J. Clin. Invest.* **38**: 484. 1959.
2. Engel, H. R., L. Hallman, S. Siegel and D. M. Bergenstal: *Proc. Soc. Exper. Biol. Med.* **98**: 753. 1958.
3. Goodman, H. M. and E. Knobil: *Endocrinology* **65**: 451. 1959.
4. Knobil, E.: *Proc. Soc. Exper. Biol. Med.* **101**: 288. 1959.
5. Raben, M. S. and V. W. Westermeyer: *Proc. Soc. Exper. Biol. Med.* **78**: 550. 1951.
6. Dole, V. P.: *J. Clin. Invest.* **35**: 150. 1956.
7. Wilhelmi, A. E.: unpublished.
8. Wilhelmi, A. E.: *The Hypophyseal Growth Hormone, Nature and Actions*, Smith, Gaebler and Long, eds. The Blakiston Division, McGraw-Hill Book Co., Inc., New York, 1955, p. 59.
9. Wilhelmi, A. E.: *Ciba Colloquia on Endocrinology*, Volume 6, G.E.W. Wolstenholme, ed., Little, Brown and Co., Boston, 1953, p. 135.
10. Lazo-Wassem, E. A., J. Michalski and C. E. Graham: *Endocrinology* **63**: 831. 1958.
11. Li, C. H.: *Fed. Proc.* **16**: 774. 1957.

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CORTICOID ACTIVITY IN THE ANDROSTANE SERIES[1]

ABSTRACT

9α-fluoro-11β-hydroxy-Δ^1-androstene-3,17-dione (FHA), an androstane derivative devoid of a side chain, has been shown to possess true corticoid activity in that it maintains the life of adrenalectomized rats and permits gain in body weight, even during stress.

Although all the typical corticoids (e.g., cortisone, cortisol, aldosterone, desoxycorticosterone) are Δ^4-unsaturated members of the pregnane (C_{21}) series, it has long been

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known that derivatives of the estrane (C_{18}) and androstane (C_{19}) series can exhibit certain corticoid-like characteristics. For example, various folliculoids and testoids produce thymolysis in the adrenalectomized rat (1, 2). Water and sodium retention, a characteristic manifestation of mineralocorticoids, can also be induced by sex hormones belonging to the estrane (2–4) and androstane (4) series, both in experimental animals and in man. Indeed, one of the common clinical complications of intense testosterone therapy is water and sodium retention (5–10). However, far from prolonging life in adrenalectomized animals, as do the true corticoids, these sex hormones are highly toxic to them and accelerate the development of deficiency symptoms (1, 2). It has recently been observed (11) that a halogenated androstane derivative, 9α-fluoro-11β-hydroxy-Δ^4-androstene-3,-17-dione (FHA), significantly decreases sodium excretion in adrenalectomized rats, although it is less active in this respect than desoxycorticosterone (DOC). FHA proved to be inactive as regards thymolytic or antiphlogistic activity in adrenalectomized rats. It did not exhibit testoid potency upon local application to the chick's comb, but stimulated the uterus of immature mice, though much less markedly than estrone. That—despite these marked deviations from the pharmacologic properties of cortisone derivatives—the compound might be a true life-maintaining corticoid had not been considered.

This possibility has now been explored, because in several earlier studies performed since the introduction of the 9α-halogenated steroids by Fried *et al.* (12), we had occasion to see that such substitution can not only increase, but even qualitatively change the actions of corticoids. In particular, it may enhance mineralocorticoid, prophlogistic and life-maintaining activity in a rather selective manner, often at the expense of antiphlogistic potency (13–15). Our first observations along these lines—briefly reported elsewhere (16)—had already shown that adrenalectomized rats can in fact be maintained alive by treatment with FHA. The object of this communication is to demonstrate that this androstane derivative also resembles true mineralocorticoids in that, even during stress, it not only maintains adrenalectomized rats, but even permits anabolism.

EXPERIMENTAL

Seventy female Sprague-Dawley rats, with a mean initial body weight of 136 gm. (range: 130–142 gm.), were bilaterally adrenalectomized and then treated as indicated in Table 1. 1% NaCl was given as a drinking fluid to the rats of Group 2 only; the other groups were maintained on tap water. All steroids were given as microcrystal suspensions at the daily dose of 400 μg. in 0.2 ml. of water, subcutaneously, desoxycorticosterone (DOC) and cortisol (COL) in the form of their acetates, 9α-fluoro-11β-hydroxy-Δ^1-androstene-3,17-dione (FHA) in unesterified form. On the 10th day 0.2 ml. of 0.5% croton oil in corn oil was injected under the plantar aponeurosis of the left hind paw of all groups, in order to produce stress. Throughout the experiment all animals were maintained exclusively on Purina Fox Chow (Ralston Purina Co., Ltd.), and on the 15th day the experiment was terminated.

RESULTS

Our principal findings are summarized in Table 1.

FHA exhibited a pronounced life-maintaining effect in these adrenalectomized rats (Group 5), even though they were exposed to the stress of a severe necrotizing croton-oil

TABLE 1. CORTICOID ACTIVITY IN THE ANDROSTANE SERIES

Group	Treatment	Final body weight (gm.)	Change in body weight (gm.)	Mortality (%)
1	None	121 ± 3.2	−15	100
2	NaCl	152 ± 9.1	+16	70
3	COL	138 ± 7.5	+ 2	0
4	DOC	195 ± 7.2	+59	20
5	FHA	171 ± 4.1	+35	0
6	COL + DOC	164 ± 2.2	+28	0
7	COL + FHA	147 ± 2.4	+11	0

arthritis. While under these circumstances all untreated controls (Group 1) and 70% of the NaCl-treated animals (Group 2) died, all FHA-injected rats survived and were manifestly in good condition at the time the experiment was discontinued. In this respect, the androstane derivative, FHA, appeared to be even more effective than DOC (Group 4) and equally effective as COL (Group 3), or COL+DOC (Group 6), although on a single dose level it would not be justified to draw definite conclusions concerning such quantitative differences in potency.

It is evident, furthermore, that in the untreated controls adrenal deficiency and stress, with the associated decrease in food-intake, led to a considerable loss of body weight; FHA caused a significant weight-increase ($P<0.01$), though less marked than DOC. The rats receiving COL alone essentially maintained their initial weight, while concurrent treatment with DOC ($P<0.01$) and—to a lesser extent—with FHA permitted some gain, but the difference in weight between Groups 3 and 7 was not statistically significant ($P>0.05$). Thus, it remains questionable whether the anti-COL action of DOC is also shared by FHA. The topical irritation arthritis—used only as a stressor in these experiments—was of such intensity that none of the steroids or steroid-combinations were able to influence its development significantly. However, additional amounts of the steroid, now being synthesized, will serve to explore the effects of FHA as a possible COL-antagonist (as regards inflammation and thymicolymphatic development); hence, these aspects of the problem need not be discussed here.

Acknowledgement

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REFERENCES

1. SCHACHER, J., J. S. L. BROWNE AND H. SELYE: *Proc. Soc. Exper. Biol. and Med.* **36**: 488. 1937.
2. SELYE, H., C. M. HARLOW AND J. B. COLLIP: *Endokrinologie* **18**: 81. 1936.
3. SELYE, H.: *Arch. Dermatol. and Syph.* **50**: 261. 1954.
4. SELYE, H.: Textbook of Endocrinology. 2nd edition. Acta, Inc., Med. Publs., Montreal. 1949.
5. PEDEN, J. C., JR., M. C. MAXWELL AND A. OHIN: *A. M. A. Arch. Surg.* **75**: 625. 1957.
6. WHEDON, G. D., E. SHORR, V. TOSCANI AND E. STEVENS: *J. Clin. Invest.* **36**: 995. 1957.
7. CALABI, V., L. GALLI AND R. RONCHI: *Minerva Med.* **49**: 1847. 1958.
8. MCSWINEY, R. R. AND F. T. G. PRUNTY: *J. Endocrinol.* **16**: 28. 1957.
9. DRILL, V. A. AND F. J. SAUNDER: Androgenic and anabolic action of testosterone derivatives. In: E. T. Engle and G. Pincus, eds., Hormones and the Aging Process. Academic Press., Inc., New York. 1956.
10. GIRAUD, G., H. LATOUR, A. LEVY, P. PUECH AND P. BARJON: *Ann. Lab. Gardiol. Clin. Méd.* p. 290, Imprimerie C. G. C., Montpellier, 1954.
11. ROSENBERG, E. AND R. I. DORFMAN: *Proc. Soc. Exper. Biol. and Med.* **99**: 336. 1958.

12. Fried, J., R. W. Thoma, D. Perlman, J. E. Herz and A. Borman: *Recent Progr. Horm. Res.* **11**: 149. 1955.
13. Selye, H.: *J. Clin. Endocrinol. and Metab.* **15**: 384. 1955.
14. Selye, H.: The Chemical Prevention of Cardiac Necroses. The Ronald Press Co., New York. 1958.
15. Selye, H. and P. Bois: *Brit. Med. J.*, Jan. 26, p. 183. 1957.
16. Selye, H.: Presented at the *IXth Internat. Congr. Pediatr. Montreal, July 21st, 1959.*

CORTICOTROPIN RELEASE FOLLOWING INJECTIONS OF MINUTE DOSES OF ARGININE VASOPRESSIN INTO THE THIRD VENTRICLE OF THE DOG[1]

ABSTRACT

Injections of 2 mU. of arginine vasopressin into the third ventricle produce a significant rise in 17-hydroxycorticosteroid level in adrenal venous blood of trained conscious dogs. In contrast, intraventricular injections of the saline vehicle or 2 mU. of oxytocin, or intravenous injections of 20 mU. of arginine vasopressin do not significantly alter 17-hydroxycorticosteroid levels. The results indicate that intraventricular injections of minute doses of arginine vasopressin induce the release of corticotropin in dogs.

Previous investigations indicate that neurohumoral substances from the hypothalamus may control corticotropin release from the anterior pituitary (1). Adrenal ascorbic acid depletion in rats (2) and a rise in plasma 17-hydroxycorticosteroids in man (3, 4) and dogs (5) have been shown to occur following injections of various posterior pituitary hormones. Increased corticotropin output was presumed to have occurred but the peripheral action of large intravenous doses of vasopressin or pitressin on adrenal corticosteroid output must be seriously considered as a possible factor in these experiments (6, 7, 8). Experimental stimulation (9, 10) and lesions in the hypothalamus (2, 11, 12) give strong evidence of a positive role of the hypothalamus in controlling corticotropin release. If a hypothetical hypothalamic neurohumoral substance reaches the anterior pituitary via the portal vessels and induces the release of corticotropin, the injection of such a substance into the third ventricle might act similarly. In the present study minute doses of arginine vasopressin were placed in the third ventricle of conscious dogs at rest and the corticosteroid output was estimated in adrenal venous blood.

MATERIALS AND METHODS

Female mongrel dogs weighing 10–14 kg. were trained to lie quietly on a board. Two operative procedures were carried out under pentobarbital anesthesia. An indwelling cannula (gauge No. 19) was implanted into the third ventricle of the brain by the technique of Bartelstone *et al.* (13). A cannula was inserted into the lumboadrenal vein according to the method of Hume and Nelson (14). Experiments were started on the second postoperative day and continued daily until thrombosis of the lumboadrenal vein occurred (3 to 5 days). During the experiments, the conscious dogs were carefully maintained in a resting state. Procaine HCl (2%) was injected locally to eliminate pain during manipulation incident to collection of adrenal venous blood. Three different solutions were used for intraventricular injections: (a) highly purified, synthetic arginine vasopressin, 2 mU. in 0.25 ml. of van Dyke-Hastings solution (15) at pH 7.4; (b) highly purified, natural oxytocin, 2 mU. in 0.25 ml. of the same vehicle, pH 7.4; (c) 0.25 ml. of the vehicle at pH 7.4. For intravenous injections, solutions containing 20 mU. of arginine vasopressin in 0.25 ml. of the above vehicle (pH 7.4) were used.

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TABLE 1. EFFECT OF INJECTIONS, EITHER INTO THE THIRD VENTRICLE OR A SUPERFICIAL VEIN, ON OUTPUT OF 17-HYDROXYCORTICOSTEROIDS IN ADRENAL VENOUS EFFLUENT

	Resting output	Peak output			
		Injection into third ventricle			Intravenous injection
		Vehicle	2 mU. oxytocin	2 mU. Arg. vasopressin	20 mU. Arg. vasopressin
No. of Determinations	22	4	6	8	4
Output (μg./min.)	2.2±0.2*	2.9±0.6*	3.1±0.5*	12.5±1.1*	3.2±1.1*
Probability†		>0.1	>0.1	<0.001	>0.05

* Mean ±Standard Error.
† Significance of Difference from Mean Resting Output.

Samples of adrenal venous blood were collected about 10 minutes or less prior to the intraventricular injection and during the following periods (minutes) after injection: 6–8, 11–13, 16–18, 21–23. The adrenal venous blood samples, 5–8 ml. each, were analyzed for 17-hydroxycorticosteroids by the Peterson modification of the Silber and Porter method (16). The dogs were protected from stress and special consideration was given to the dogs' physical and psychological status before and during the experimental runs. Any changes in environmental conditions were carefully noted.

RESULTS

The adrenal output of 17-hydroxycorticosteroids following third ventricular injections is summarized in Table 1. Following injections of the vehicle or 2 mU. oxytocin there is no significant change in the corticosteroid output compared to resting levels (average 2.2 μg. per minute). Intraventricular injections of 2 mU. arginine vasopressin

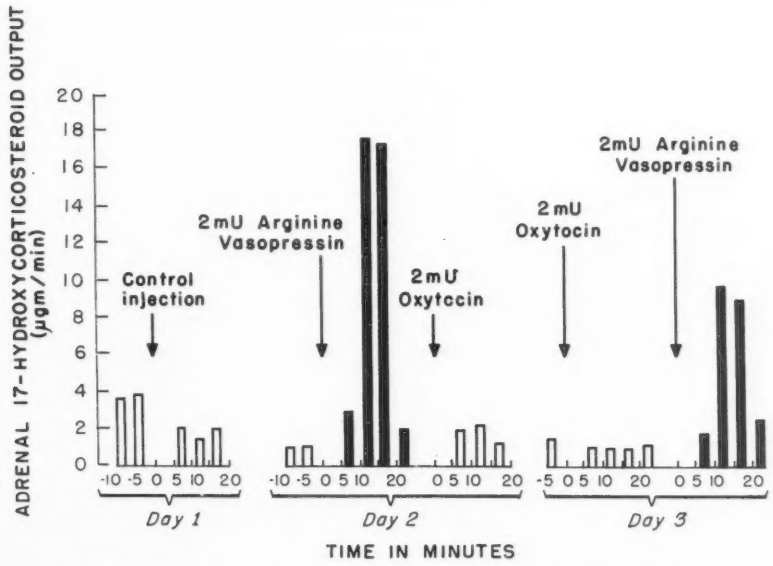

FIG. 1. Adrenal corticosteroid output following injections into the third ventricle of a conscious dog. The control injection consisted of 0.25 ml. of van Dyke-Hastings solution, pH 7.4.

are consistently followed by a significant rise in adrenal corticosteroid output. The peak rise averages 12.5 μg. per minute. Intravenous injection of 20 mU. arginine vasopressin does not significantly change corticosteroid output.

The corticosteroid output obtained from a typical experimental animal is illustrated in Figure 1. It should be noted that the sequence of injections of vasopressin and oxytocin was reversed on successive days. The pattern of response remains unaltered.

High corticosteroid values were obtained (5–8 μg./min.) prior to intraventricular injections when the dogs were under the stress of pain, fever, infection, blood loss, or untoward laboratory conditions produced by sounds, unfamiliar personnel and the presence of other animals. The results of a given day's experiments were rejected therefore if resting values indicated that the dog had been disturbed. If altered conditions induced stress during an experimental run, a persistent rise in corticosteroid output occurred (10–15 μg./min.) which must not be confused with the transient response to intraventricular vasopressin.

DISCUSSION

Intraventricular injections of 2 mU. of arginine vasopressin caused a definite increase in adrenal 17-hydroxycorticosteroid output which closely resembles the pattern of response which follows intravenous injection of corticotropin in hypophysectomized dogs (17). Intraventricular injections of 2 mU. of oxytocin, or the vehicle alone, fail to increase adrenal corticosteroid output. Intravenous injection of 10 times the intraventricular dose of arginine vasopressin does not affect adrenal corticosteroid levels. 2 mU. of arginine vasopressin placed in the third ventricle did not, therefore, cause increased corticosteroid secretion through a peripheral action.

The release of corticosteroids following the intraventricular injection of arginine vasopressin does not necessarily prove it to be the physiological corticotropin releasing factor. The conditions under which intraventricular vasopressin acts are currently being investigated. Vasopressin analogues are also under study.

Acknowledgments

The highly purified neurohypophysial hormones used were kindly furnished by Prof. V. du Vigneaud. The authors are indebted to Prof. H. B. van Dyke for his guidance during these experiments.

Addendum

Since submission of the manuscript, a total of eight intraventricular injections of 2 mU. arginine vasopressin, in 4 additional dogs, produced results similar to those of Table 1. Furthermore, 5 μμg. of either arginine vasotocin (arginine oxytocin) or oxypressin (leucine vasopressin) when injected into the third ventricle did not produce any significant increase in adrenal corticosteroid output.

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REFERENCES

1. DONOVAN, B. T. AND G. W. HARRIS: *Ann. Rev. Physiol.* **19**: 439. 1957.
2. MCCANN, S. M. AND J. R. BROBECK: *Proc. Soc. Exper. Biol. & Med.* **87**: 318. 1954.
3. MCDONALD, R. K. AND V. K. WEISE: *Proc. Soc. Exper. Biol. & Med.* **92**: 107. 1956.

[2] Fellow of the China Medical Board of New York, Inc.

4. McDonald, R. K. and V. K. Weise: *Proc. Soc. Exper. Biol. & Med.* **92:** 481. 1956.
5. Nichols, B., Jr. and R. Guillemin: *Endocrinology* **64:** 914. 1959.
6. Hume, D. M. and D. H. Nelson: *Proc. Endocrine Soc. Meet.* **39:** 98. New York, 1957.
7. Royce, P. C. and G. Sayers: *Proc. Soc. Exper. Biol. & Med.* **98:** 70. 1958.
8. Hilton, J. G., L. F. Scian, C. D. Westermann and O. R. Kruesi: *Fed. Proc.* **18:** 68. 1959.
9. Porter, R. W.: *Recent Progress in Hormone Research* **10:** 1. New York, 1954.
10. Mason, J. W.: *Endocrinology* **63:** 403. 1958.
11. Hume, D. M.: *Ann. Surg.* **138:** 548. 1953.
12. Daily, W. J. R. and W. F. Ganong: *Endocrinology* **62:** 442. 1958.
13. Bartelstone, H. J., H. A. Reilly and S. C. Wang: *J. Appl. Physiol.* **13:** 142. 1958.
14. Hume, D. M. and D. H. Nelson: *Surg. Forum* **5:** 568. 1955.
15. van Dyke, H. B. and A. B. Hastings: *Proc. Soc. Exper. Biol. & Med.* **27:** 748. 1930.
16. Peterson, R. E., A. Karrer and S. L. Guerra: *Anal. Chem.* **29:** 144. 1957.
17. Nelson, D. H. and D. M. Hume: *Endocrinology* **57:** 184. 1955.

PROGRAM OF THE FIRST INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

THE program of the First International Congress of Endocrinology, which will be held in Copenhagen, Denmark, July 18–23, 1960, will consist of ten symposia and a round table discussion, along with sessions of communicated papers. The speakers at the symposia and the round table discussion have been invited by the Subcommittee on Program of the Congress. In symposia #1–8 and in #10, the Subcommittee has also invited a discusser for each principal paper. Symposium #9 will consist of six principal papers and no discussers. The round table discussion will consist of eight sections, each with a principal speaker and three secondary speakers. This program of invited speakers is as follows:

1. *Central nervous system regulation of anterior pituitary secretion.* July 19, 9–12 A.M.
   J. Benoit, France: Système nerveux central et contrôle de la fonction gonadotrope préhypophysaire. Discusser: S. Katsuki, Japan.
   G. Harris, England: Central nervous control of gonadotrophic and thyrotrophic secretion. Discusser: L. Desclin, Belgium.
   H. D. Purves, New Zealand: Hypothalamic control of thyrotrophin and prolactin secretion. Discusser: J. J. van der Werff ten Bosch. Netherlands.
   G. Sayers, U.S.A.: Mediation of hypothalamic control over corticotropin release. Discusser: R. J. Guillemin, U.S.A.
2. *Posterior pituitary hormones.* July 19, 1–3 P.M.
   H. Croxatto, Chile: Natural and synthetic related substances having neurohypophyseal hormone-like activities. Discusser: R. A. Boissonas, Switzerland.
   R. Caldeyro-Barcia, Uruguay: Oxytocin in pregnancy and labor. Discusser: F. Stutinski, France.
   H. Heller, England: Antidiuretic hormones of the neurohypophysis. Discusser: L. Martini, Italy.
3. *Parathyroid hormone and calcium metabolism.* July 19, 3:30–5:30 P.M.
   H. Rasmussen, U.S.A.: The nature and properties of the parathyroid hormone. Discusser: P. Munson, U.S.A.
   W. F. Neuman, U.S.A.: Experimental biology of the parathyroid secretions. Discusser: P. J. Gaillard, Netherlands.
   T. R. Fraser, England: Clinical tests of parathyroid abnormality. Discusser: F. C. Bartter, U.S.A.
4. *Anterior pituitary hormones.* July 20, 9–12 A.M.
   A. B. Lerner, U.S.A.: The mechanism of action of the melanocyte stimulating hormone. Discusser: K. Hofmann, U.S.A.
   C. H. Li, U.S.A.: Biochemical endocrinology of pituitary growth hormone. Discusser: M. S. Raben, U.S.A.
   R. Luft, Sweden: Studies on the metabolic action of human growth hormone. Discusser: J. Gillman, South Africa.
   J. A. Loraine, Scotland: Gonadotrophin assays as an index of pituitary function in man. Discusser: C. A. Gemzell, Sweden.

5. *Adrenocortical syndromes.* July 20, 1–3 P.M.
D. J. Ingle, U.S.A.: The relationship of the adrenal glands to diseases of adaptation. Discusser: T. Symington, Scotland.
T. F. Gallagher, U.S.A.: Studies of steroid metabolism in man. Discusser: T. F. G. Prunty, England.
L. Wilkins, U.S.A.: The treatment of virilizing adrenal hyperplasia. Discusser: J. Decourt, France.
6. *Aldosterone.* July 20, 3:30–5:30 P.M.
G. Farrell, U.S.A.: Influence of the mesencephalon and epiphysis cerebri on adrenal function. Discusser: A. F. Muller, Switzerland.
F. Gross, Switzerland: Regulation and mechanism of sodium transport by aldosterone and related corticoids. Discusser: H. P. Wolff, Germany.
J. W. Conn, U.S.A.: The evolution of primary aldosteronism from 1954 to 1960. Clinical manifestations, pathology, and the results of adrenal surgery. Discusser: F. Tronchetti, Italy.
7. *Aspects of reproduction.* July 22, 9–12 A.M.
A. Jost, France: The effect of sex-related steroids on embryonic sex differentiation and growth. Discusser: C. W. Emmens, Australia.
H. Nowakowski, Germany: Congenital disorders of the testis. Discusser: J. Charvat, Czechoslovakia.
E. Diczfalusy, Sweden: Endocrine functions of the human placenta. Discusser: J. Zander, Germany.
G. Pincus, U.S.A: Fertility control by endocrine agents. Discusser: W. O. Nelson, U.S.A.
8. *Steroid pharmacology.* July 22, 1–3 P.M.
A. Zaffaroni, Mexico: The effect of alkyl- and electronegative group substitution on steroidal hormonal activity. Discusser: M. Tausk, Netherlands.
R. Hertz, U.S.A: The pharmacological alteration of the biosynthesis and the peripheral effects of steroid hormones. Discusser: T. Nakao, Japan.
J. Ferin, Belgium: Comparative assays of new sex steroids in the human. Discusser: G. Sala, Italy.
9. *Hormone action at the cellular level.* July 22, 3:30–5:30 P.M.
R. Michel, France: Pénétration et utilisation cellulaire des hormones thyroïdiennes.
P. Talalay, U.S.A.: The participation of steroid hormones in enzymatic reactions.
O. Hechter, U.S.A.: Cell permeability and hormone action.
E. W. Sutherland, Jr., U.S.A.: Formation of cyclic 3,5-adenylic acid and its relation to the action of several hormones.
P. J. Randle, England: Interactions between metabolism and sugar transport in muscle as a site of insulin action.
N.-Å. Hillarp, Sweden: Storage and release of catechol amines.
10. *Topics in comparative endocrinology.* July 23, 9–12 A.M.
O. Mühlbock, Netherlands: Hormonal factors in tumorigenesis and tumor growth. Discusser: O. Pearson, U.S.A.
C. M. Williams, U. S. A.: Present understanding of the juvenile hormone of insects. Discusser: P. Karlson, Germany.
L. Gallien, France: Etude comparée des activités des hormones stéroïdes dans la différenciation sexuelle des amphibiens. Problèmes de specificité et effets paradoxaux. Discusser: F. Pautsch, Poland.
N. L. Gerbilskii, U.S.S.R.: Endocrinology of fishes. Discusser: E. C. Amoroso, England.

Round Table Discussion: The validity of hormone assays applicable to clinical medicine. July 23, afternoon.

1. Corticosteroids. R. H. Silber, U.S.A.; secondary speakers: J. K. Norymberski, England; R. I. Peterson, U.S.A.; R. V. Brooks, England.
2. Androgens. R. I. Dorfman, U.S.A.; secondary speakers: C. J. Migeon, U.S.A.; L. O. Plantin, Sweden; A. E. Kellie, England.
3. Aldosterone. J. F. Tait, U.S.A.; secondary speakers: R. Neher, Switzerland; E. H. Venning, Canada; J. A. Luetscher, Jr., U.S.A.
4. Progesterone, pregnanediol, and pregnanetriol. R. Borth, Switzerland; secondary speakers: A. Klopper, Scotland; R. V. Short, England; A. M. Bongiovanni, U.S.A.
5. Gonadotrophins. A. Albert, U.S.A.; secondary speakers: C. Hamburger, Denmark; B. Lunenfeld, Israel; R. Henry, France.
6. Estrogens. J. B. Brown, Scotland; secondary speakers: J. R. K. Preedy, U.S.A.; M. F. Jayle, France; G. Ittrich, Germany.
7. Thyroid hormones and thyrotrophin. A Querido, Netherlands; secondary speakers: J. B. Stanbury, U.S.A.; P. M. Bottari, Belgium; J. E. Rall, U.S.A.
8. Catechol amines. U. S. von Euler, Sweden; secondary speakers: F. Elmadjian, U.S.A.; M. D. Armstrong, U.S.A.; H. Weil-Malherbe, U.S.A.

Investigators interested in presenting papers to the communicated sessions are reminded that abstract forms for such papers must be secured from the Congress Secretariat, Statens Seruminstitut, Copenhagen S, Denmark, and that these abstracts must be submitted by December 31, 1959.

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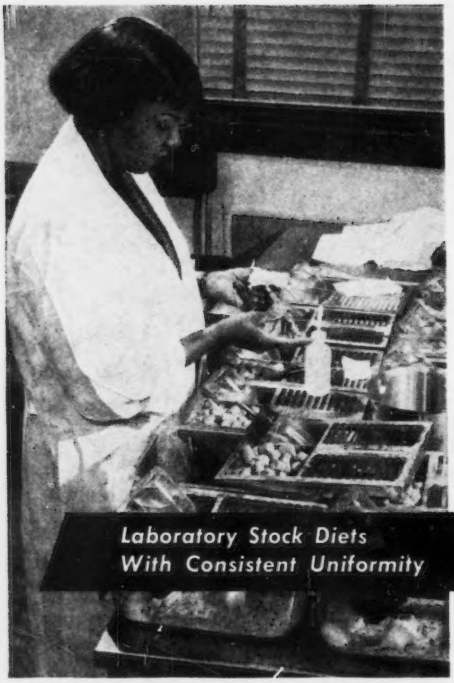

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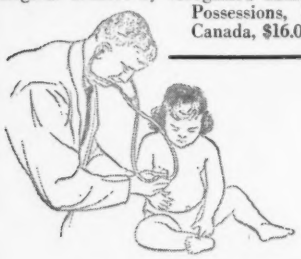

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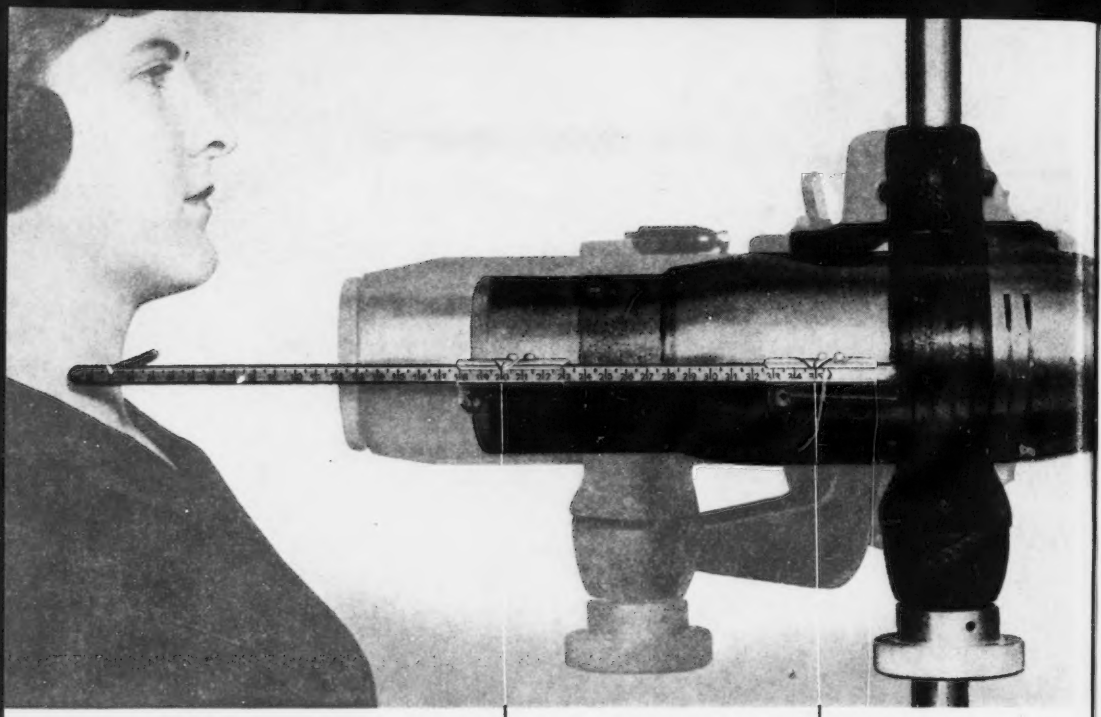

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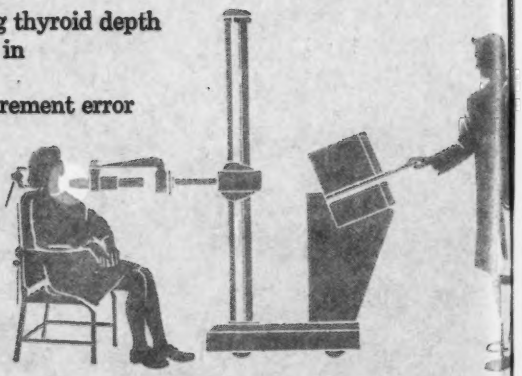

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